PEPTIDE NUCLEIC ACID CONJUGATES

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of United States application serial number 08/108,591, filed November 27, 1993 that, in turn, is a national phase application of PCT application EP/01219, filed May 22, 1992, claiming priority to Danish Patent Applications: No. 986/91, filed May 24, 1991, No. 987/91, filed May 24, 1991, and No. 510/92, filed April 15, 1992. In addition, this application is a continuation-in-part to United States application serial number 08/088,658, filed July 2, 1993, United States application serial number 08/088,661, filed July 2, 1993 and United States application serial number 08/275,951, filed July 15, 1994.

The contents of the foregoing patent applications are incorporated herein by reference.

FIELD OF THE INVENTION

This invention is directed to compounds that are not polynucleotides yet which bind to complementary DNA and RNA strands more strongly than the corresponding DNA. In particular, the invention concerns peptide nucleic acids (PNAs) which are functionalized to include covalently bound conjugates.

BACKGROUND OF THE INVENTION

Oligonucleotides and their analogs have been developed and used in molecular biology in certain procedures as probes, primers, linkers, adapters, and gene fragments. Modifications to oligonucleotides used in these procedures include labeling with non isotopic labels, e.g. fluorescein, biotin, digoxigenin, alkaline phosphatase, or other reporter molecules. Other modifications have been made to the ribose phosphate backbone to

increase the resistance to nucleases. These modifications include use of methyl phosphonates, phosphorothioates, phosphorodithioate linkages, and 2'-O-methyl ribose sugar units. Further modifications include those made to modulate uptake and cellular distribution. Phosphorothioate oligonucleotides are presently being used as antisense agents in human clinical trials for the treatment of various disease states, and as antiviral agents. With the success of these oligonucleotides for both diagnostic and therapeutic uses, there exists an ongoing demand for improved oligonucleotide analogs.

Oligonucleotides can interact with native DNA and RNA in several ways. For example, an oligonucleotide may form a duplex with a single stranded nucleic acid, or form a triplex structure by binding to double stranded DNA. However, to form a triplex structure with a double stranded DNA, the cytosine bases of the oligonucleotide must be protonated. Triplexing is therefore pH dependent. P.O.P. Ts'o and associates have used pseudo isocytosine as a permanently protonated analogue of cytosine in DNA triplexing (see Ono, *et al.*, *J. Am. Chem. Soc.*, **1991**, *113*, 4032-4033; Ono, *et. al.*, *J. Org. Chem.*, **1992**, *57*, 3225-3230). Trapane and Ts'o have also suggested the use of pseudo isocytosine for triplex formation with single-stranded nucleic acid targets. (see, Trapane, *et. al.*, *J. Biomol. Strul. Struct.*, **1991**, *8*, 229; Trapane, *et. al.*, *Biophys. J.*, **1992**, *61*, 2437; and Trapane, *et. al.*, *Abstracts Conference on Nucleic Acids Medical Applications*, Cancun, Mexico, January 1993). WO 93/05180 discloses substitution of 8-Oxoadenine for protonated cytosine in triplex formation.

Peptide nucleic acids (PNAs) are compounds that in some respects are analogous to oligonucleotides, but which differ in structure. In peptide nucleic acids, the deoxyribose backbone of oligonucleotides has been replaced with a backbone having peptide linkages. Each subunit has attached a naturally occurring or non-naturally occurring base. One such backbone is constructed of repeating units of N-(2-aminoethyl)glycine linked through amide bonds.

PNA binds both DNA and RNA to form PNA/DNA or PNA/RNA duplexes. The resulting PNA/DNA or PNA/RNA duplexes are bound with greater affinity than corresponding DNA/DNA or DNA/RNA duplexes as evidence by their higher melting temperatures (Tm). This high thermal stability has been attributed to the neutrality of the

PNA backbone, which does not encounter the charge repulsion present in DNA or RNA duplexes. The neutral backbone of the PNA also renders the Tms of PNA/DNA(RNA) duplexs practically independent of salt concentration. Thus the PNA/DNA duplex offers a further advantage over DNA/DNA duplex interactions which are highly dependent on ionic

5   strength. Homopyrimidine PNAs have been shown to bind complementary DNA or RNA forming (PNA)2/DNA(RNA) triplexes of high thermal stability (*see, e.g.*, Egholm, *et al.*, Science, **1991**, *254*, 1497; Egholm, *et al.*, J. Am. Chem. Soc., **1992**, *114*, 1895; Egholm, *et al.*, J. Am. Chem. Soc., **1992**, *114*, 9677).

In addition to increased affinity, PNA has also been shown to bind to DNA with

10   increased specificity. When a PNA/DNA duplex mismatch is melted relative to the DNA/DNA duplex there is seen an 8 to 20 °C drop in the Tm. This magnitude of a drop in Tm is not seen with the corresponding DNA/DNA duplex with a mismatch present. *See* Egholm, M., et al., *Nature* **1993** *365* p. 566.

The binding of a PNA strand to a DNA or RNA strand can occur in one of two

15   orientations. The orientation is said to be anti-parallel when the DNA or RNA strand in a 5' to 3' orientation binds to the complementary PNA strand such that the carboxyl end of the PNA is directed towards the 5' end of the DNA or RNA and amino end of the PNA is directed towards the 3' end of the DNA or RNA. In the parallel orientation the carboxyl end and amino end of the PNA are in reverse orientation with respect to the 5'-3' direction of the

20   DNA or RNA.

PNAs bind to both single stranded DNA and double stranded DNA. It has been observed that two strands of PNA can bind to dsDNA. While PNA/DNA duplexes are stable in the antiparallel configuration, it was previously believed that the parallel orientation is preferred for (PNA)2/DNA triplexes.

25   The binding of two single stranded pyrimidine PNAs to a double stranded DNA has been shown to occur via strand displacement, rather than by conventional triple helix formation as observed with triplexing oligonucleotides. When PNA strands invade double stranded DNA, one strand of the DNA is displaced and forms a loop on the side of the PNA2/DNA complex area. The other strand of the DNA is part of the (PNA)2/DNA triplex

30   structure. The single stranded loop area (known as a D loop) is susceptible to cleavage by

enzymes that can cleave single stranded DNA.

A further advantage of PNA compared to oligonucleotides is that their polyamide backbone is not recognized by either nucleases or proteases, and are therefore resistant to degradation by enzymes.

5   Because of their properties, PNAs are known to be useful in several different applications.   Since PNAs have stronger binding and greater specificity than oligonucleotides, they are of great utility as probes in cloning, blotting procedures, and in applications such as fluorescence *in situ* hybridization (FISH). Homopyrimidine PNAs are used for strand displacement in homopurine targets. The restriction sites that overlap with

10   or are adjacent to the D-loop will not be cleaved by restriction enzymes. Additionally, the local triplex inhibits gene transcription. The binding of PNAs to specific restriction sites within a DNA fragment can inhibit cleavage at those sites. This inhibition is useful in cloning and subcloning procedures. Labeled PNAs are also used to directly map DNA molecules by hybridizing PNA molecules having a fluorescent label to complementary

15   sequences in duplex DNA using strand invasion.

PNAs also have been used to detect point mutations in PCR-based assays (PCR clamping). In PCR clamping, PNA is used to detect point mutations in a PCR-based assay, e.g. the distinction between a common wild type allele and a mutant allele, in a segment of DNA under investigation. Typically, a PNA oligomer complementary to the wild type

20   sequence is synthesized and included in the PCR reaction mixture with two DNA primers, one of which is complementary to the mutant sequence. The wild type PNA oligomer and the DNA primer compete for hybridization to the target. Hybridization of the DNA primer and subsequent amplification will only occur if the target is a mutant allele. With this method, the presence and exact identity of a mutant can be determined.

25   Considerable research is being directed to the application of oligonucleotides and oligonucleotide analogs that bind complementary DNA and RNA strands for use as diagnostics, research reagents and potential therapeutics.   For many uses, the oligonucleotides and oligonucleotide analogs must be transported across cell membranes or taken up by cells to express activity.

30   PCT/EP/01219 describes novel peptide nucleic acid (PNA) compounds which bind

complementary DNA and RNA more tightly than the corresponding DNA. It is desirable to append to these compounds groups which modulate or otherwise influence their activity or their membrane or cellular transport. One method for increasing such transport is by the attachment of a pendant lipophilic group. United States application serial number 117,363, filed Sept. 3, 1993, entitled "Amine-Derivatized Nucleosides and Oligonucleosides", describes several alkylamino functionalities and their use in the attachment of such pendant groups to oligonucleosides.

Additionally, United States application serial number 07/943,516, filed Sept. 11, 1992, entitled "Novel Amines and Methods of Making and Using the Same" and corresponding published PCT application WO 94/06815 describe other novel amine-containing compounds and their incorporation into oligonucleotides for, *inter alia*, the purposes of enhancing cellular uptake, increasing lipophilicity, causing greater cellular retention and increasing the distribution of the compound within the cell.

United States application serial number 08/116,801, filed Sept. 3, 1993, entitled "Thiol-Derivatized Nucleosides and Oligonucleosides" describes nucleosides and oligonucleosides derivatized to include thiolalkyl functionality, through which pendant groups are attached.

There remains a need in the art for stable compounds that bind complementary DNA and RNA to form double-stranded, helical structures which mimic double-stranded DNA, and which are capable of being derivatized to bear pendant groups to further enhance or modulate their binding, cellular uptake, or other activity.

OBJECTS OF THE INVENTION

It is an object of the invention to provide PNAs having at least one conjugate attached thereto.

It is a further object of this invention to provide PNAs which include alkylamino chemical functionality.

It is another object of this invention to provide PNAs which include alkylthio chemical functionality.

It is a further object of the invention to provide PNAs having improved transfer

properties across cellular membranes.

It is another object to provide peptide nucleic acids that include intercalators, nucleic acid cleaving agents, cell surface phospholipids, and/or diagnostic agents.

These and other objects will become apparent from the following description and

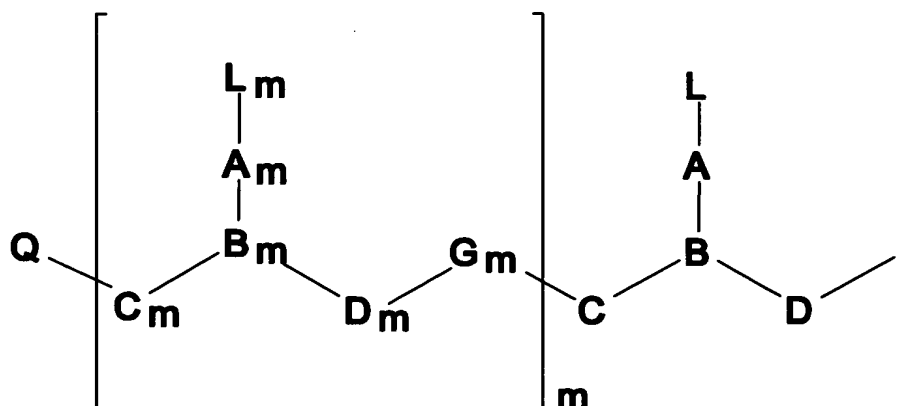5   accompanying claims.

SUMMARY OF THE INVENTION

The present invention provides PNA conjugates comprising a PNA backbone and a

10   conjugate bound to the PNA either directly or through a linking moiety. The PNA backbone has an amino end, a carboxyl end, and a plurality of amido groups. Nucleobases are tethered to the backbone through internally located amino groups.

The conjugate is bound through the linking moiety to at least one of the backbone, the tether, or the nucleobase. Preferably, the conjugate is bound to the backbone.

15   The conjugate can be a reporter enzyme, a reporter molecule, a steroid, a carbo-hydrate, a terpene, a peptide, a protein, an aromatic lipophilic molecule, a non aromatic lipophilic molecule, a phospholipid, an intercalator, a cell receptor binding molecule, a crosslinking agent, a water soluble vitamin, a lipid soluble vitamin, an RNA/DNA cleaving complex, a metal chelator, a porphyrin, an alkylator, or a polymeric compound selected

20   from polymeric amines, polymeric glycols and polyethers. The conjugate is preferably bound to the peptide nucleic acid backbone, a tether or a nucleobase through a linking moiety.

Also provided are PNA conjugates of the formula:

$$\left[\begin{array}{c} \underset{\displaystyle A_m}{\overset{\displaystyle L_m}{|}} \\ Q \diagdown \underset{\displaystyle C_m}{\overset{\displaystyle B_m}{|}} \diagdown \underset{\displaystyle D_m}{} \diagdown \underset{\displaystyle C}{\overset{\displaystyle G_m}{}} \end{array} \right]_m \underset{\displaystyle D}{\overset{\displaystyle \underset{\displaystyle B}{\overset{\displaystyle A}{\overset{\displaystyle |}{}}}}{}} \diagdown I$$

wherein:

    m is an integer from 1 to about 50;

5   L and L_m independently are $R^{12}(R^{13})_a$; wherein:

        R^{12} is hydrogen, hydroxy, $(C_1\text{-}C_4)$alkanoyl, a naturally occurring nucleobase, a non-naturally occurring nucleobase, an aromatic moiety, a DNA intercalator, a nucleobase-binding group, a heterocyclic moiety, a reporter ligand, or a conjugate;

10   provided that at least one of R^{12} is a naturally occurring nucleobase, a non-naturally occurring nucleobase, a DNA intercalator, or a nucleobase-binding group;

        R^{13} is a conjugate; and

        a is 0 or 1;

15   C and C_m independently are $(CR^6R^7)_y$; wherein:

        R^6 and R^7 independently are hydrogen, a side chain of a naturally occurring alpha amino acid, $(C_2\text{-}C_6)$ alkyl, aryl, aralkyl, heteroaryl, hydroxy, $(C_1\text{-}C_6)$ alkoxy, $(C_1\text{-}C_6)$ alkylthio, a conjugate, NR^3R^4, SR^5 or R^6 and R^7 taken together complete an alicyclic or heterocyclic system;

20   wherein R^5 is hydrogen, a conjugate, $(C_1\text{-}C_6)$alkyl, hydroxy-, alkoxy-, or alkylthio- substituted $(C_1\text{-}C_6)$alkyl; and

        R^3 and R^4 independently are hydrogen, a conjugate, $(C_1\text{-}C_4)$alkyl,

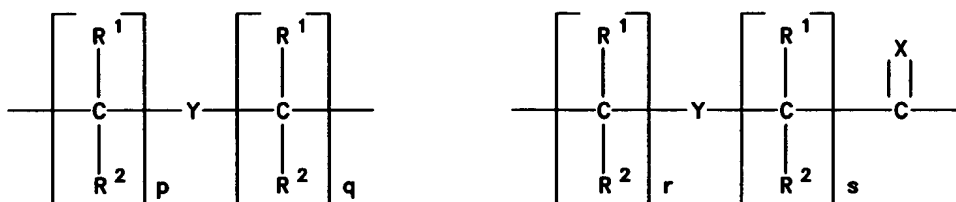hydroxy- or alkoxy- or alkylthio-substituted (C_1-C_4)alkyl, hydroxy, alkoxy, alkylthio or amino;

D and D_m independently are $(CR^6R^7)_z$;

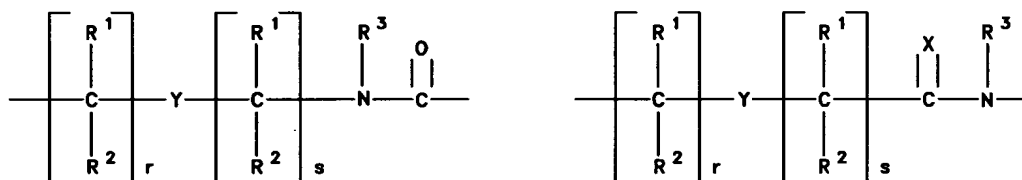each of y and z is zero or an integer from 1 to 10, wherein the sum $y + z$ is greater than 2 but not more than 10;

G_m is independently $-NR^3CO-$, $-NR^3CS-$, $-NR^3SO-$, or $-NR^3SO_2-$ in either orientation;

each pair of A-A_m and B-B_m are selected such that:

(a) A or A_m is a group of formula (IIa), (IIb) or (IIc) and B or B_m is N or R^3N^+; or

(b) A or A_m is a group of formula (IId) and B or B_m is CH;

$$\left[\begin{array}{c} R^1 \\ | \\ -C- \\ | \\ R^2 \end{array} \right]_p \; Y \left[\begin{array}{c} R^1 \\ | \\ -C- \\ | \\ R^2 \end{array} \right]_q \qquad\qquad \left[\begin{array}{c} R^1 \\ | \\ -C- \\ | \\ R^2 \end{array} \right]_r \; Y \left[\begin{array}{c} R^1 \\ | \\ -C- \\ | \\ R^2 \end{array} \right]_s \begin{array}{c} X \\ \| \\ -C- \end{array}$$

5

$$\left[\begin{array}{c} R^1 \\ | \\ -C- \\ | \\ R^2 \end{array} \right]_r \; Y \left[\begin{array}{c} R^1 \\ | \\ -C- \\ | \\ R^2 \end{array} \right]_s \begin{array}{c} R^3 \quad O \\ | \quad\; \| \\ -N-C- \end{array} \qquad\qquad \left[\begin{array}{c} R^1 \\ | \\ -C- \\ | \\ R^2 \end{array} \right]_r \; Y \left[\begin{array}{c} R^1 \\ | \\ -C- \\ | \\ R^2 \end{array} \right]_s \begin{array}{c} X \quad R^3 \\ \| \quad\; | \\ -C-N- \end{array}$$

10

wherein:

X is O, S, Se, NR^3, CH_2 or $C(CH_3)_2$;

15   Y is a single bond, O, S or NR^4;

each of p and q is zero or an integer from 1 to 5, the

sum p+q being not more than 10;

each of r and s is zero or an integer from 1 to 5, the sum r+s being not more than 10;

R^1 and R^2 independently are hydrogen, $(C_1$-$C_4)$alkyl, hydroxy-substituted $(C_1$-$C_4)$alkyl, alkoxy-substituted $(C_1$-$C_4)$alkyl, alkylthio-substituted $(C_1$-$C_4)$alkyl, hydroxy, alkoxy, alkylthio, amino, halogen or a conjugate;

I is -NR^8R^9 or -$NR^{10}C(O)R^{11}$; wherein:

R^8, R^9, R^{10} and R^{11} independently are hydrogen, alkyl, an amino protecting group, a reporter ligand, an intercalator, a chelator, a peptide, a protein, a carbohydrate, a lipid, a steroid, a nucleoside, a nucleotide, a nucleotide diphosphate, a nucleotide triphosphate, an oligonucleotide, an oligonucleoside, a soluble polymer, a non-soluble polymer or a conjugate;

Q is -CO_2H, -CO_2R^8, -CO_2R^9, -$CONR^8R^9$, -SO_3H, -$SO_2NR^{10}R^{11}$ or an activated derivative of -CO_2H or -SO_3H; and
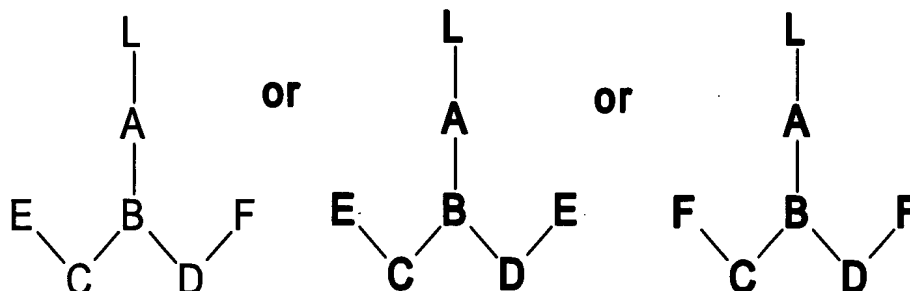
wherein at least one of R^1, R^2, R^3, R^4, R^5, R^6, R^7, R^8, R^9, R^{10}, R^{11}, R^{12} and R^{13} is a conjugate wherein said conjugate is a reporter enzyme, a reporter molecule, a steroid, a carbohydrate, a terpene, a peptide, a protein, an aromatic lipophilic molecule, a non aromatic lipophilic molecule, a phospholipid, an intercalator, a cell receptor binding molecule, a crosslinking agent, a water soluble vitamin, a lipid soluble vitamin, an RNA cleaving complex, a metal chelator, a porphyrin an alkylator, or a polymeric compound selected from polymeric amines, a polymeric glycols and polyethers; and wherein the conjugate optionally includes a linking moiety. The conjugate is preferably bond to the PNA through a linking moiety.

In preferred embodiments at least one group R^1, R^2, R^3, R^4, R^5, R^6, R^7, R^8, R^9, R^{10}, R^{11} R^{12} or R^{13} is a conjugate. In other preferred embodiments at least one of group A-A_m includes at least one of group R^1, R^2 or R^3.

At least one of groups B-B_m or groups G-G_m preferably include at least one group R^3, and at least one of said groups Q or I preferably include at least one of groups R^8, R^9, R^{10} and R^{11}. At least one of said groups D-D_m, or C-C_m preferably include at least one of R^3, R^4, R^5, R^6 and R^7.

In preferred embodiments m is from 1 to about 200, preferably from 1 to about 50, and more preferably from 1 to about 20.

Also provided are compounds having one of the following formulas:

$$
\begin{array}{ccc}
\begin{array}{c}
L \\
| \\
A \\
| \\
E \diagdown \; B \diagup F \\
C \quad D
\end{array}
&
\text{or}
\begin{array}{c}
L \\
| \\
A \\
| \\
E \diagdown \; B \diagup E \\
C \quad D
\end{array}
&
\text{or}
\begin{array}{c}
L \\
| \\
A \\
| \\
F \diagdown \; B \diagup F \\
C \quad D
\end{array}
\end{array}
$$

5

wherein:

L, A, B, C and D are as defined above.

E independently is COOH, CSOH, SOOH, SO_2OH or an activated or protected derivative thereof;

10   F independently is NHR^3 or $NPgR^3$, where Pg is an amino protecting group; and

at least one of R^1, R^2, R^3, R^4, R^5, R^6, R^7, R^{12}, and R^{13} is a conjugate as defined above, and wherein the conjugate is preferably bound through a linking moiety.

In preferred embodiments at least one of R^3, R^4, R^5, R^6, and R^7 is a conjugate. In other preferred embodiments at least one group R^3 is a conjugate. Preferably, R^{12} is a

15   conjugate or R^{13} is a conjugate. In some preferred embodiments at least one group A or group B includes a conjugate, or at least one of said groups C or said groups D include a conjugate.

Also provided are peptide nucleic acid conjugates comprising a plurality of PNA monomers wherein at least one of the PNA monomers has the formula:
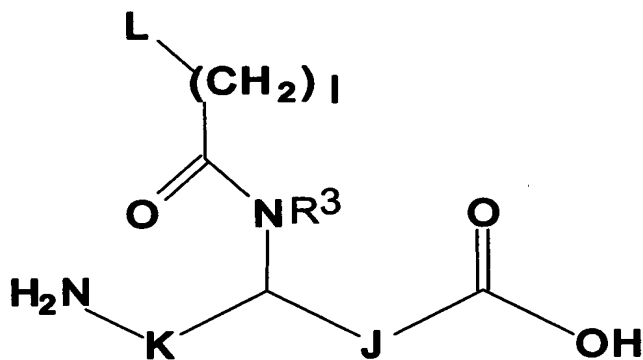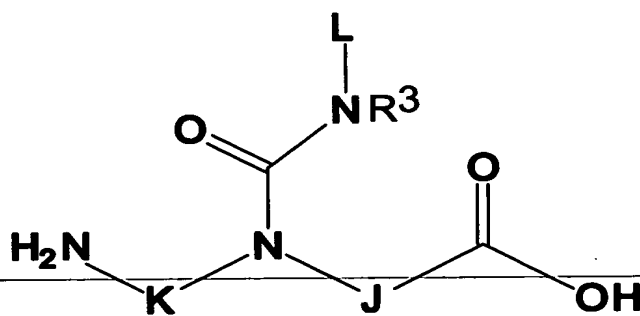
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or formula:

15
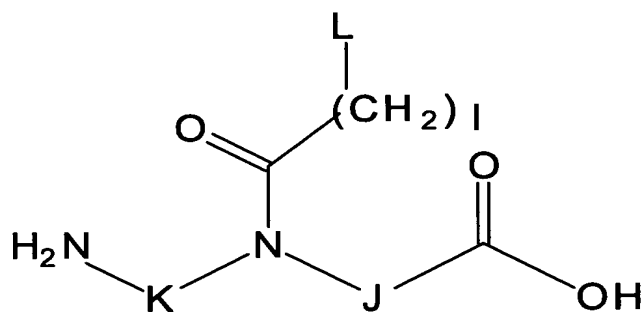
25

or formula:

30

wherein:

L is defined as above;

K is $(CR^6R^7)_z$ and J is $(CR^6R^7)_y$ wherein R^6, R^7, y and z are defined above; l is an integer from 1 to 5; and

at least one of R^1, R^2, R^3, R^4, R^5, R^6, R^7, R^{12}, and R^{13} is a conjugate as defined above. Preferably, at least one of group K, group J or R^3 includes a conjugate, preferably bound through a linking moiety.

BRIEF DESCRIPTION OF THE FIGURES

The numerous objects and advantages of the present invention may be better understood by those skilled in the art by reference to the accompanying figures, in which:

Figure 1 is a reaction schematic depicting the exemplary functionalization of the 2-aminoethyl portion of a PNA backbone at the 1-position.

Figure 2 is a reaction schematic depicting the exemplary functionalization of the 2-aminoethyl portion of a PNA backbone at the 2-position.

Figure 3 depicts exemplary routes for chemical alteration of monomer synthons of the invention.

Figure 4 is a reaction schematic depicting alternate exemplary routes useful in the practice of the invention.

Figure 5 is a reaction schematic depicting the exemplary functionalization of the OH group of an L-serine portion of a PNA backbone.

Figure 6 is a reproduction of an electrophoresis gel pertinent to Example 111, showing that PNA NTA $Fe2^+$ is an effective oligonucleotide cleavage agent.

DETAILED DESCRIPTION OF THE INVENTION

This invention is directed to novel peptide nucleic acids having at least one conjugate group. Each PNA monomer or polymer has a backbone which is the linear portion of the PNA. The backbone is normally defined from an amino (N) terminus or deravative thereof to a carboxyl (C) terminus or derivative thereof. In some preferred

embodiments the backbone has an N terminal end, a C terminal end, a number of amide linkages which is equal to one less than the number of monomer units in the PNA, and a number of internal substituted amine groups which is equal to the number of monomers in the PNA.

5   The N terminal or an internal amide nitrogen of the PNA backbone is separated from the nearest substituted amino group by a number of methylene groups which may bear substituent groups. In preferred embodiments there are two such methylene groups. The substituted amino group is bound to a tether, which is in turn bound to a ligand. The ligand is typically a nucleobase.

10   The substituted amino group is separated from the C terminal carboxyl group or an internal peptidyl carbonyl group of the PNA backbone by one or more methylene groups which may bear substituent groups. In preferred embodiments there is one such methylene group. Thus, in these preferred embodiments the PNA backbone of n monomeric units has the structure:

15         $H-[HN-(CH_2)_2-N(tether-nucleobase)-(CH_2)-CO]_n-OH$

wherein the methylene groups optionally bear substituent groups in place of hydrogen.

Monomers and oligomers of the present invention include at least one conjugate attached to the N terminal end, C terminal end, a backbone methylene (inclusive of all methylenes in the backbone), an internal substituted amine, a tether, a ligand or an amide

20   linkage between monomers in an oligomer.

As used herein the term nucleobase includes naturally occurring heterocyclic bases such as adenine, guanine, thymine, cytosine and uracil, and also non-naturally occurring nucleobase analogs, homologs and modified nucleobases such as those bearing removable protecting groups. Some representative nucleobase ligands and illustrative synthetic ligands

25   are shown in Figure 2 of WO 92/20702.

As will be recognized, a variety of PNA conjugates can be prepared according to the present invention. Representative conjugates can be formed from homopolymeric PNA strands or heteropolymeric PNA strands (e.g., chimeras of PNA-DNA or PNA-RNA). Each PNA strand or PNA portion of a chimeric strand preferably includes a plurality of ligands, L

30   (typically nucleobases) linked to a backbone via attachment at the position found in nature,

i.e., position 9 for adenine or guanine, and position 1 for thymine or cytosine. Alternatively, the ligand can be a non-naturally occurring nucleobase (a nucleobase analog), another base-binding moiety, an aromatic moiety, (C_1-C_4)alkanoyl, hydroxy or hydrogen. L also can be further substituted with a conjugate attached through an optional linking group. In some

5   preferred embodiments L is a conjugate. In monomer synthons, L can be blocked with protecting groups, as illustrated in Figure 4 of WO 92/20702.

The term "conjugate" as used herein includes a reporter enzyme, a reporter molecule, a steroid, a carbohydrate, a terpene, a peptide, a protein, an aromatic lipophilic molecule, a non aromatic lipophilic molecule, a phospholipid, an intercalator, a cell receptor

10   binding molecule, a crosslinking agent, a water soluble vitamin, a lipid soluble vitamin, an RNA cleaving complex, a metal chelator, a porphyrin, an alkylator, and polymeric compounds such as polymeric amines, polymeric glycols and polyethers. PNAs of the present invention include at least one conjugate attached directly or through an optional linking moiety. When so derivatized, the PNA is useful, for example, as a diagnostic or

15   therapeutic agent, to render other properties to a complementary nucleic acid in a test structure or to transfer a therapeutic or diagnostic agent across cellular membranes. Such a diagnostic or therapeutic agent is formed from an oligomeric compound of the invention wherein the oligomeric compound includes monomeric units bearing natural or non-natural occurring nucleobases in a sequence that is complementary to and will specifically

20   hybridize with a region of an RNA or DNA of interest. Thus, an oligomeric compound of the invention is "functionalized" to include a conjugate attached to the oligomeric compound via an optional linking moiety. For the purpose of identification, such a functionalized oligomeric compound can be characterized as a substituent-bearing (*e.g.*, steroid-bearing) oligomeric compound. Such oligomeric compounds will have at least one

25   conjugate attached thereto to modulate their activity.

The oligomeric compounds of the present invention may be useful in binding to various other target molecules. Target molecules of the present invention can include any of a variety of biologically significant molecules. Target molecules can include but are not limited to nucleic acids, carbohydrates, glycoproteins or other proteins.

30   The PNA oligomers and monomer synthons of the invention are constructed such

that conjugates may be covalently bound thereto. In one aspect of the invention conjugates are molecular entities appended to PNAs which serve to modulate, optimize, alter or otherwise affect the activity, transport, uptake or longevity of the desired PNA.

For the purposes of this invention the term "modify" shall mean to change properties

5   by addition to, subtraction from, cleavage of or otherwise, such that which results is intrinsically different from that which is modified. The term "modulate" shall mean to change (*i.e.*, increase or decrease) the magnitude of a property.

A PNA monomer can be functionalized and derivatized to include a conjugate, and the resulting conjugated monomer can be incorporated into a PNA oligomer. Alternatively,

10   one or more conjugates can be incorporated into an oligomeric PNA that has been functionalized at predetermined positions. It will be apparent to those skilled in the art that a wide range of chemistries for the attachment of conjugates are amenable to the present invention.

Tether A can be a wide variety of groups such as

15    $-CR^1R^2C(O)-$, $-CR^1R^2C(S)-$, $-CR^1R^2C(Se)-$, $-CR^1R^2C(O)N(R^3)-$, $-CR^1R^2C(CH_2)-$ and $-CR^1R^2C[C(CH_3)_2]-$, where R^1, R^2 and R^3 are as defined above. Preferably, A is methylenecarbonyl ($-CH_2CO-$), amido ($-CONR^3-$), or, taken together with B when B is R^3N^+, ureido ($-N^+R^3CONR^3-$). A also can be a longer chain moiety such as propanoyl, butanoyl or pentanoyl, or a corresponding derivative wherein O is replaced by

20   another moiety for X or the chain is substituted with R^1R^2, or contains Y where Y is a heteroatom. Further, A can be a (C_2-C_6)alkylene chain, a (C_2-C_6)alkylene chain substituted with R^1R^2 or contains Y where Y is a heteroatom. In some embodiments A is a single bond.

In one preferred embodiment B is a nitrogen atom, thereby presenting the possibility

25   of an achiral backbone. B can also be CH or R^3N^+, where R^3 is as defined above.

In a preferred embodiment C is $-CR^6R^7-$ or a two carbon unit, *i.e.* $-CHR^6CHR^7-$ or $-CR^6R^7CH_2-$, where R^6 and R^7 are as defined above. R^6 and R^7 also can be a heteroaryl group such as, for example, pyrrolyl, furyl, thienyl, imidazolyl, pyridyl, pyrimidinyl, indolyl, or can be taken together to complete an alicyclic system such as, for example, 1,2-

30   cyclobutanediyl, 1,2-cyclopentanediyl or 1,2-cyclohexanediyl. C can also be used as a point

of attachment of a conjugate e.g. at R^6 and or R^7. In some preferred embodiments G is -$CONR^3$-. In monomeric embodiments F can be NHR^3 or $NPgR^3$ where Pg is an amino protecting group, and E can be CSOH, SOOH, SO_2OH or an activated derivative thereof. In polymeric embodiments groups E and F are activated and chemically combined to

5   produce group G, which therefore can be -$CSNR^3$-, -$SONR^3$- or -SO_2NR^3-. The activation can, for example, be achieved using an acid anhydride or an active ester derivative, wherein hydroxy in the groups represented by E is replaced by a leaving group suited for generating the growing backbone.

In some cases it may be useful to attach conjugates at either terminus (Q, I) to modu-

10   late the binding characteristics of the resulting PNAs. Representative conjugates include DNA intercalators, which improve dsDNA binding and increase cellular uptake, and basic groups such as lysine or polylysine, which strengthen the binding of the PNA by electrostatic interaction. Other moieties can be located on non-terminal positions. Oligonucleotides and/or oligonucleosides can be covalently bound to terminal positions Q

15   or I to form chimeras containing PNA portions and oligonucleotide and/or oligonucleoside portions. Nucleosides and/or nucleotides (mono, di or tri-phosphates) also can be attached to the terminal positions.

Figure 1 is a reaction schematic depicting the exemplary functionalization of the 2-aminoethyl portion of a PNA backbone at the 1 position. Starting with the commercially

20   available (O-benzyl)Boc-Serine, the acid is protected as the methyl ester using diazomethane and MeOH, yielding the fully protected Compound **30A**. Compound **30A** is reduced at the ester to give the Compound **30B** having a free hydroxyl which is converted to the protected amine Compound **30C** using standard Mitsunobo chemistry. Compound **30C** is reacted with methyl bromoacetate to form a monomeric backbone unit which is further

25   reacted with a chlorocarbonyl functionalized nucleobase or other ligand to form the functionalized monomer with side chain protected as an O-benzyl. The phthalimido group is removed and the free amine is protected with a BOC group, and the monomer is attached to MBHA Lysine resin to yield the initial monomer attached to the solid support. The support-bound monomer can then be deprotected using TFA, and reacted with a second

30   functionalized monomer or unfunctionalized monomer to give the dimer. The process is

repeated in an iterative fashion until the desired PNA is assembled.

Figure 2 is a reaction schematic depicting the exemplary functionalization of the 2-aminoethyl portion of a PNA backbone at the 2-position. Compound **30C** is deprotected with hydrazine and ethanol to give Compound **34A**, which is further reacted with methyl

5   bromoacetate to yield the functionalized backbone Compound **34B**. Compound **34B** is reacted with an acetyl chloride activated nucleobase or other ligand to form the functionalized monomer with the sidechain protected as a benzyl ether. Compound **34** results when 1-thyminyl-acetylchloride is used as the acetyl chlorocarbonyl functionalized nucleobase. Compound **34** is then attached to a suitable resin as shown in Figure 1. The

10   support-bound monomer is then deprotected with TFA, and reacted with additional monomers to yield the desired PNA chain.

Figure 3 depicts the transformation of PNA monomer sidechains. Compound **30** is converted from the phthalimido protected amine to the Boc protected amine using hydrazine and $Boc_2O/NaOH$, and the benzyl protected hydroxyl is deprotected by hydrogenolysis to

15   yield Compound **35**, which may participate in activation and displacement reactions typical of hydroxyl groups. Thus, PNA oligomer and monomer synthons of the invention may be functionalized with the many chemical species which are useful in the derivatization of DNA and RNA 2'- and 3'-hydroxyls.

Thus, linking moieties attached to PNA monomers of the invention can be modified

20   through a variety of processes, and may participate in linkages with a wide variety of conjugates or conjugates that have themselves been functionalized with a linking moiety.

Compound **35** may also be oxidized to the carbonyl (aldehyde) compound **37A**. Compound **37A** may participate in Schiff's base linkages, which may be stabilized by reductive amination with, for example, sodium borohydride. Compound **37A** may also be

25   further functionalized via enolate condensations, organometallic reactions, stabilized Wittig reactions, and other reactions of active carbonyl groups which will be readily apparent to those skilled in the art.

Figure 4 depicts an alternate route to the functionalized backbone. The commercially available BOC-serine is reacted with triphenylphosphine/DEAD and then

30   TFA/TsOH to produce Compound **46A**. Compound **46A** is treated with Gabriel's reagent to

give Compound **46B**. Protection of the amine function as the phthalimide and esterification with ethanol gives Compound **46C**. Deprotection with TFA gives Compound **46D**, which can be used in the preparation of PNA monomers functionalized at the 2 position of the 2-aminoethyl portion of the monomer, as shown in Figure 1. Deprotection with hydrazine

5   gives Compound **46E**, which can be used in the preparation of PNA monomers functionalized at the 1 position of the 2-aminoethyl portion of the monomer, as shown in Figure 2.

It will be seen that selective deprotection of Compound **46C** with either hydrazine or TFA allows the synthesis of monomers functionalized at position 1 or 2 of the 2-aminoethyl

10   portion of the monomer.

Functionalization of the backbone at another position is illustrated in Figure 5. 3-(Boc-amino)-1,3-propanediol is converted to N-Boc-aminoacetaldehyde Compound **39A** via periodate oxidation. Treatment of Compound **39A** according to the procedures of Method A, with glacial acetic acid, and sodium triacetoxyborohydride will give the

15   intermediate Compound **39B**. Compound **39B** can have a variety of different functionalities attached to the secondary amino group in a like manner to Compound **30D** and **34B**. Compound **39B** is further reacted with 1-carboxymethylthyminyl (compound **3**) in 1N NaOH to give Compound **39**. The benzyl-protected serine hydroxyl of compound **39** is deprotected according to the procedures of Example **42**, and oxidized to a formyl group as

20   per the procedures of Example **43**. The formyl compound is further functionalized to yield the aminoethyl compound as per the procedures of Example **44**. Compound **44** can be treated in a number of different ways to yield other compounds of the invention.

In one aspect of the present invention Compound **44** is treated with a protecting group, incorporated into a PNA oligomer, deprotected and further functionalized with a

25   conjugate that may include an optional linking moiety. In another aspect of the present invention Compound **44** is functionalized with a tether and then incorporated into a PNA oligomer. After oligomerization the protecting group is removed and the tether is reacted with a conjugate that may include an optional linking moiety. In a further aspect of the invention the conjugate which may include an optional linking moiety is attached to

30   Compound **44** prior to oligomerization. It will be understood by those skilled in the arts

that many variations of the methods and techniques described above are amenable to the present invention.

It will also be seen that for the PNAs of the invention it may be advantageous to incorporate one or more functionalized monomers into the chain. The monomers may be independently functionalized at position 1 or 2 of the 2-aminoethyl portion of the monomer as illustrated above or at some other position e.g. O-lysine as illustrated above.

The term PNA oligomer as used herein means linked PNA monomers. The monomers that make up the oligomers are further referred to as synthons. A preferred linkage is an amide linkage between consecutive PNA monomers. Conjugates may be attached to a given PNA by a chemical bond to the PNA backbone as illustrated above, to ligand L, or to tether A. The term "PNA backbone" as used herein is intended to mean the linear polymeric chain of the PNA.

The amino acids which form the backbone can be identical or different. Those based on 2-aminoethylglycine are especially well suited to the purpose of the invention. Another backbone is the 2-aminoethylserine benzyl ether. Preferred are those backbones which are functionalized to include linking moieties capable of participating in linkages to conjugates, or to other linking moieties which are in turn attached to conjugates.

Thus, monomer synthons according to the invention may be prepared with linking moieties contained therein, and the desired conjugate group may be bound to the linking moiety either before or after assembly into a PNA oligomer. It will be seen that not every monomer synthon need be functionalzed. However, if advantageous, all the monomers in a given PNA oligomer may be so functionalized.

Conjugates are attached to a linking moiety, a PNA monomer, or a PNA oligomer by the formation of a chemical bond from the reaction of two functional groups. Representative functional groups for formation of the chemical bond include but are not limited to hydroxy, formyl, carboxy or other active carbon species, amino, substituted amino, thio, sulfoxo, sulfono, sulfinic or sulfate ester, or other species similarly able to participate in a chemical linkage with a conjugate group. Functional groups also include those which are protected or "masked." Protecting groups are known *per se* as chemical functional groups that can be selectively appended to and removed from functionalities such

as amine groups and thiol groups. These groups are present in a chemical compound to render such functionality inert to chemical reaction conditions to which the compound is exposed. *See, e.g.*, Greene and Wuts, *Protective Groups in Organic Synthesis*, 2d ed, John Wiley & Sons, New York, 1991.

5   In some instances it is advantageous to link conjugate groups to PNA oligomers or monomer synthons through a linking moiety. Representative linking moieties useful in the practice of the invention are disclosed in United States application serial number 08/116,801, filed Sept. 3, 1993, entitled "Thiol-Derivatized Nucleosides and Oligonucleosides" and United States application serial number 117,363, filed Sept. 3, 1993,

10   entitled "Amine-Derivatized Nucleosides and Oligonucleosides", the disclosures of which are hereby incorporated by reference.

Thus, the monomer synthons (and therefore ultimately the PNA oligomers) of the invention may be constructed containing any one of several functionalities for the linking of conjugate groups, or linkers attached to conjugate groups (i.e., carboxy, formyl, thio,

15   hydroxy, amino and the like). Those skilled in the art will appreciate that the PNA monomer and oligomers of the invention are capable of conjugation to a wide variety of linking functionalities. For example, to attach a conjugate containing a free amino group to a PNA monomer synthon or PNA oligomer, one may functionalize monomers to contain a carboxyl group, and create an amide linkage using standard chemistries. Similarly, in some

20   instances it might be advantageous to attach the amino group through a PNA monomeric unit functionalized with a formyl moiety by reductive amination as described in the Example **85**. In general, the choice of a particular functional group on a monomer synthon will be driven by the available functional groups on the conjugate group or linking molecule. Those skilled in the art will be able to determine the optimal match of

25   functionalities by application of standard principles.

One aspect of the present invention is directed to PNAs that bear at least one thiol-containing linking moiety at a PNA backbone, tether or ligand position previously described. Representative thiol linking moieties useful for attaching a conjugate to PNA backbone, ligand or tether positions are -NH-$(CH_2)_6$-S-H and -CH_2-O-$(CH_2)_6$-S-H where H

30   can be optionally replaced with a protecting group. It can readily be seen that a large

number of variations on these linking moieties are possible. Numerous thiol protecting groups are known in the art, including, but not limited to, the triphenylmethyl (trityl; Tr) and S-t-butyl, S-p-nitrobenzyl, and S-p-methoxy-benzyl (*see, e.g.*, Greene and Wuts, Protective Groups in Organic Synthesis, 2d edition, John Wiley & Sons, New York, 1991).

5   Preferred linker groups according to the invention include an alkyl moiety independently selected to having 1 to about 12 carbon atoms or alkyl groups interspaced with suitable heteroatoms including oxygen, sulfur and nitrogen to form polyethers, polythioethers or polyalkylamines. The term "alkyl" is intended to include straight chain and branched hydrocarbons. In such linkers, the preferred length of these hydrocarbons is 1

10   to about 7 carbon atoms. Other linkers include heterobifunctional and homobifunctional linkers.

For the purposes of this invention the terms "reporter molecule" and "reporter enzyme" are inclusive of those molecules or enzymes that have physical or chemical properties that allow them to be identified in gels, fluids, whole cellular systems, broken

15   cellular systems and the like utilizing physical properties such as spectroscopy, radio-activity, colorimetric assays, fluorescence, and specific binding. Particularly useful as reporter molecules are biotin, rhodamine, coumarin and dye molecules including fluorescein dyes. Particularly useful as reporter enzymes are alkaline phosphatase and horseradish peroxidase.

20   Steroids include those chemical compounds that contain a perhydro-1,2-cyclopentanophenanthrene ring system. Particularly useful as steroid molecules are the bile acids including cholic acid, deoxycholic acid and dehydrocholic acid; steroids including cortisone, digoxigenin, testosterone and cholesterol and even cationic steroids such as cortisone having a trimethylaminomethyl hydrazide group attached via a double bond at the

25   3-position of the cortisone ring.

Proteins and peptides are utilized in their usual sense as polymers of amino acids. Normally peptides comprise such polymers that contain a smaller number of amino acids per unit molecule than do the proteins. Particularly useful as peptides and proteins are sequence-specific peptides and proteins including phosphodiesterase, peroxidase,

30   phosphatase and nuclease proteins. Such peptides and proteins include but are not limited

to SV40 peptide, RNase A, RNase H and Staphylococcal nuclease.

Lipophilic molecules include naturally-occurring and synthetic aromatic and non-aromatic moieties such as fatty acids, esters, alcohols and other lipid molecules, cage structures such as adamantane and buckminsterfullerenes, and aromatic hydrocarbons such

5   as benzene, perylene, phenanthrene, anthracene, naphthalene, pyrene, chrysene, and naphthacene. Particularly useful as lipophilic molecules are alicyclic hydrocarbons, saturated and unsaturated fatty acids, waxes, terpenes and polyalicyclic hydrocarbons including adamantane and buckminsterfullerenes. Particularly useful as terpenoids are vitamin A, retinoic acid, retinal and dehydroretinol.

10 Alkylators according to the invention are moieties that can effect attachment of electrophilic groups to targeted molecular structures. Representative alkylators are disclosed by Meyer, *et al.*, *J. Am. Chem. Soc.* **1989**, *111*, 8517.

Intercalators are polycyclic aromatic moieties that can insert between adjacent base pairs without affecting normal Watson-Crick base pairing. Representative intercalators are

15 pyrene, phenanthroline, phenazine, phenanthridine, anthraquinone, acridine, and these and other examples are further disclosed by Manoharan, M., *Antisense Research and Applications*, Crooke and Lebleu, eds., CRC Press, Boca Raton, 1993. Pyrenes include pyrene and other pyrene-based carboxylic acids that can be conjugated using standard protocols. Intercalators of the present invention also include hybrid intercalators. One

20 example of a hybrid intercalator is the photonuclease/intercalator 6-[[[9-[[6-(4-nitrobenz-amido)hexyl]amino]acridin-4-yl]carbonyl]amino]hexanoyl-pentafluorophenyl ester. This compound has two noteworthy features: an acridine moiety that is an intercalator and a p-nitro benzamido group that is a photonuclease.

Cell receptor binding molecules according to the invention are vitamins and

25 carbohydrate moieties for which specific receptors exist within a cell. Representative cell receptor binding molecules are disclosed by Application Serial No. PCT/US92/09196, filed October 23, 1992, the contents of which are incorporated herein by reference.

Crosslinking agents are moieties that can effect intrastrand or interstrand covalent binding of RNA and/or DNA. Representative crosslinking agents are disclosed in Inter-

30 national Patent Application Serial No. PCT/US93/02059, filed March 5, 1993, which is

incorporated herein by reference. Peptide nucleic acids are disclosed herein and by International Patent Application WO 92/20702, published November 26, 1992.

Crosslinking agents of the present invention include photo-crosslinking agents. One group of photo-crosslinking agents is the aryl azides e.g. N-hydroxysuccinimidyl-4-azidobenzoate (HSAB) and N-succinimidyl-6(-4'-azido-2'-nitrophenyl-amino)hexanoate (SANPAH). Aryl azides conjugated to PNA will effect crosslinking with nucleic acids and proteins upon irradiation. They will also crosslink with carrier proteins (such as KLH or BSA), raising an antibody against the oligonucleotides.

Vitamins according to the invention generally can be classified as water soluble or lipid soluble. Water soluble vitamins include thiamine, riboflavin, nicotinic acid or niacin, the vitamin B_6 pyridoxal group, pantothenic acid, biotin, folic acid, the B_{12} cobamide coenzymes, inositol, choline and ascorbic acid. Lipid soluble vitamins include the vitamin A family, vitamin D, the vitamin E tocopherol family and vitamin K (and phytols). The vitamin A family, including retinoic acid and retinol, are absorbed and transported to target tissues through their interaction with specific proteins such as cytosol retinol-binding protein type II (CRBP-II), Retinol-binding protein (RBP), and cellular retinol-binding protein (CRBP). These proteins, which have been found in various parts of the human body, have molecular weights of approximately 15 kD. They have specific interactions with compounds of vitamin-A family, especially, retinoic acid and retinol.

The vitamin A family of compounds can be attached to PNA oligomers of the invention via acid or alcohol functionalities found in the various family members. For example, conjugation of an N-hydroxy succinimide ester of an acid moiety of retinoic acid to an amine function of a PNA or a linker attached to a PNA results in linkage of vitamin A to the PNA via an amide bond.

α-Tocopherol (vitamin E) and the other tocopherols (beta through zeta) can be conjugated to PNAs to enhance uptake because of their lipophilic character. Also, the lipophilic vitamin, vitamin D, and its ergosterol precursors can be attached to PNAs through their hydroxyl groups by first activating the hydroxyl groups to, for example, hemisuccinate esters. Conjugation then is effected to an aminolinker from the PNA, or to the PNA backbone through the other suitable functional groups described herein. Other vitamins that

can be conjugated to PNAs or PNA aminolinkers through hydroxyl groups on the vitamins include thiamine, riboflavin, pyridoxine, pyridoxamine, pyridoxal, deoxypyridoxine. Lipid soluble vitamin K's and related quinone-containing compounds can be conjugated via carbonyl groups on the quinone ring. The phytol moiety of vitamin K may also serve to

5   enhance binding of the PNA to cells.

Pyridoxal (vitamin B_6) has specific B_6-binding proteins. The role of these proteins in pyridoxal transport has been studied by Zhang and McCormick, *Proc. Natl. Acad. Sci. USA*, **1991** *88*, 10407. Zhang and McCormick also have shown that a series of N-(4'-pyridoxyl)amines, in which several synthetic amines were conjugated at the 4'-position of

10   pyridoxal, are able to enter cells by a process facilitated by the B6 transporter. They also demonstrated the release of these synthetic amines within the cell. Other pyridoxal family members include pyridoxine, pyridoxamine, pyridoxal phosphate, and pyridoxic acid. Pyridoxic acid, niacin, pantothenic acid, biotin, folic acid and ascorbic acid can be conjugated to PNAs using N-hydroxysuccinimide esters that are reactive with the PNA or

15   aminolinkers located on the PNA, as described above for retinoic acid.

Other groups for modifying properties of PNAs include RNA/DNA cleaving complexes. RNA/DNA cleavers include *o*-phenanthroline/metal complexes and Ru(bipyridine)$_3^{2+}$ complexes. The Ru(bpy)$_3^{2+}$ complexes interact with nucleic acids and cleave nucleic acids photochemically. Metal chelators include EDTA, DTPA, nitrilo-

20   triacetate(NTA), and o-phenanthroline. Porphyrins include porphine, its substituted forms, and metal complexes.

A polyamine or polymeric amine species as used herein refers to species that have a plurality of nitrogen atoms thereon. Polyamines include primary amines, hydrazines, semicarbazines, thiosemicarbazines and similar nitrogenous species. Such species can be

25   symmetrical species such as polyamine containing polymers or they can be unsymmetrical wherein the amine functionalities of the polyamine are separated in space by different moieties. In addition to carbon atoms other atomic species such as nitrogen and sulfur may also be incorporated into the polyamine species. In some preferred embodiments of the invention, at least one nitrogen atom of the polyamine has a free electron pair.

30   Preferred as polyamines are species that range in length from about 3 to about 20

units. More preferably species having at least one nitrogen atom have the general formula $H_2N[(CH_2)_nNH]_m$- wherein n is an integer between 2 and 8 and m is an integer between 1 and 10. These species can be linear or cyclic. Cyclic amines would include crown amines and mixed crown amines/crown ethers.

5   Other suitable nitrogen-containing compounds suitable for the formation of polyamines include C_1-C_{20} straight chain alkylamine, C_1-C_{20} straight chain substituted alkylamine, C_2-C_{50} branched chain alkylamine, C_2-C_{50} branched chain substituted alkylamine, C_3-C_{50} cyclic alkylamine, C_3-C_{50} cyclic substituted alkylamine, C_2-C_{20} straight chain alkenylamine, C_2-C_{20} straight chain substituted alkenylamine, C_3-C_{50} branched chain

10   alkenylamine, C_3-C_{50} branched chain substituted alkenylamine, C_3-C_{50} cyclic alkenylamine, C_3-C_{50} cyclic substituted alkenylamine, C_2-C_{20} straight chain alkynylamine, C_2-C_{20} straight chain substituted alkynylamine, C_3-C_{50} branched chain alkynylamine, C_3-C_{50} branched chain substituted alkynylamine, C_3-C_{50} cyclic alkynylamine, C_3-C_{50} cyclic substituted alkynylamine, C_1-C_{20} straight chain alkylhydrazine, C_1-C_{50} straight chain substituted

15   alkylhydrazine, C_2-C_{50} branched chain alkylhydrazine, C_2-C_{50} branched chain substituted alkylhydrazine, C_3-C_{50} cyclic hydrazoalkane, C_3-C_{50} cyclic substituted hydrazoalkane, C_2-C_{20} straight chain alkenylhydrazine, C_2-C_{20} straight chain substituted alkenylhydrazine, C_3-C_{50} branched chain alkenylhydrazine, C_3-C_{50} branched chain substituted alkenylhydrazine, C_3-C_{50} cyclic hydrazoalkene, C_3-C_{50} cyclic substituted hydrazoalkene, C_2-C_{20} straight chain

20   alkynylhydrazine, C_2-C_{20} straight chain substituted alkynylhydrazine, C_3-C_{50} branched chain alkynylhydrazine, C_3-C_{50} branched chain substituted alkynylhydrazine, C_3-C_{50} cyclic hydrazoalkyne, C_3-C_{50} cyclic substituted hydrazoalkyne, C_1-C_{20} straight chain alkylhydroxyamine, C_1-C_{20} straight chain substituted alkylhydroxyamine, C_2-C_{50} branched chain alkylhydroxyamine, C_2-C_{50} branched chain substituted alkylhydroxyamine, C_3-C_{50}

25   cyclic oxyalkylamine, C_3-C_{50} cyclic substituted oxyalkylamine, C_2-C_{20} straight chain alkenylhydroxyamine, C_2-C_{20} straight chain substituted alkenylhydroxyamine, C_3-C_{50} branched chain alkenylhydroxyamine, C_3-C_{50} branched chain substituted alkenylhydroxyamine, C_3-C_{50} cyclic oxyalkenylamine, C_3-C_{50} cyclic substituted oxyalkenylamine, C_2-C_{20} straight chain alkynylhydroxyamine, C_2-C_{20} straight chain

30   substituted alkynylhydroxyamine, C_3-C_{50} branched chain alkynylhydroxyamine, C_3-C_{50}

branched chain substituted alkynylhydroxyamine, C_3-C_{50} cyclic oxyalkynylamine, C_3-C_{50} cyclic substituted oxyalkynylamine, C_1-C_{20} straight chain alkylsemicarbazide, C_1-C_{20} straight chain substituted alkylsemicarbazide, C_2-C_{50} branched chain alkylsemicarbazide, C_2-C_{50} branched chain substituted alkylsemicarbazide, C_3-C_{50} cyclic alkylsemicarbazide,

5   C_3-C_{50} cyclic substituted alkylsemicarbazide, C_2-C_{20} straight chain alkenylsemicarbazide, C_2-C_{20} straight chain substituted alkenylsemicarbazide, C_3-C_{50} branched chain alkenylsemicarbazide, C_3-C_{50} branched chain substituted alkenylsemicarbazide, C_3-C_{50} cyclic alkenylsemicarbazide, C_3-C_{50} cyclic substituted alkenylsemicarbazide, C_2-C_{20} straight chain alkynylsemicarbazide, C_2-C_{20} straight chain substituted alkynylsemicarbazide, C_3-C_{50}

10   branched chain alkynylsemicarbazide, C_3-C_{50} branched chain substituted alkynylsemicarbazide, C_3-C_{50} cyclic alkynylsemicarbazide, C_3-C_{50} cyclic substituted alkynylsemicarbazide, C_1-C_{20} straight chain alkylthiosemicarbazide, C_1-C_{20} straight chain substituted alkylthiosemicarbazide, C_2-C_{50} branched chain alkylthiosemicarbazide, C_2-C_{50} branched chain substituted alkylthiosemicarbazide, C_3-C_{50} cyclic alkylthiosemicarbazide,

15   C_3-C_{50} cyclic substituted alkylthiosemicarbazide, C_2-C_{20} straight chain alkenylthiosemicarbazide, C_2-C_{20} straight chain substituted alkenylthiosemicarbazide, C_3-C_{50} branched chain alkenylthiosemicarbazide, C_3-C_{50} branched chain substituted alkenylthiosemicarbazide, C_3-C_{50} cyclic alkenylthiosemicarbazide, C_3-C_{50} cyclic substituted alkenylthiosemicarbazide, C_2-C_{20} straight chain alkynylthiosemicarbazide, C_2-C_{20} straight

20   chain substituted alkynylthiosemicarbazide, C_3-C_{50} branched chain alkynylthiosemicarbazide, C_3-C_{50} branched chain substituted alkynylthiosemicarbazide, C_3-C_{50} cyclic alkynylthiosemicarbazide, C_3-C_{50} cyclic substituted alkynylthiosemicarbazide, C_1-C_{20} straight chain alkylhydrazone, C_1-C_{20} straight chain substituted alkylhydrazone, C_2-C_{50} branched chain alkylhydrazone, C_2-C_{50} branched chain substituted alkylhydrazone, C_3-

25   C_{50} cyclic hydrazoalkane, C_3-C_{50} cyclic substituted hydrazoalkane, C_2-C_{20} straight chain alkenylhydrazone, C_2-C_{20} straight chain substituted alkenylhydrazone, C_3-C_{50} branched chain alkenylhydrazone, C_3-C_{50} branched chain substituted alkenylhydrazone, C_3-C_{50} cyclic hydrazoalkene, C_3-C_{50} cyclic substituted hydrazoalkene, C_2-C_{20} straight chain alkynylhydrazone, C_2-C_{20} straight chain substituted alkynylhydrazone, C_3-C_{50} branched

30   chain alkynylhydrazone, C_3-C_{50} branched chain substituted alkynylhydrazone, C_3-C_{50} cyclic

hydrazoalkyne, C_3-C_{50} cyclic substituted hydrazoalkyne,C_1-C_{20} straight chain alkylhydrazide, C_1-C_{20} straight chain substituted alkylhydrazide, C_3-C_{50} branched chain alkylhydrazide, C_3-C_{50} branched chain substituted alkylhydrazide, C_3-C_{50} cyclic alkylhydrazide, C_3-C_{50} cyclic substituted alkylhydrazide, C_2-C_{20} straight chain

5   alkenylhydrazide, C_2-C_{20} straight chain substituted alkenylhydrazide, C_3-C_{50} branched chain alkenylhydrazide, C_3-C_{50} branched chain substituted alkenylhydrazide, C_3-C_{50} cyclic alkenylhydrazide, C_3-C_{50} cyclic substituted alkenylhydrazide,C_2-C_{20} straight chain alkynylhydrazide, C_2-C_{20} straight chain substituted alkynylhydrazide, C_3-C_{50} branched chain alkynylhydrazide, C_3-C_{50} branched chain substituted alkynylhydrazide, C_3-C_{50} cyclic

10   alkynylhydrazide and C_3-C_{50} cyclic substituted alkynylhydrazide.

In accordance with preferred embodiments of the present invention polyamines are linear or cyclic non-aromatic polyamines. A useful class of cyclic non-aromatic polyamines are crown amines which are disclosed by Studer, *et al.*, *Helv. Chim. Acta* **1986**, *69*, 2081 and Smith-Jones, *et al.*, *Bioconjugate Chem.* **1991**, 2, 415. In still more preferred

15   embodiments of the present invention polyamines are linear or cyclic non-aromatic comprising non-amide nitrogen atoms. By non-amide is meant a nitrogen which is not adjacent to a carbonyl group (i.e. C=O or C=S).

Representative polyethylene glycol (PEG)-containing groups are disclosed by Ouchi, *et al.*, *Drug Design and Discovery* **1992**, *9*, 93, Ravasio, *et al.*, *J. Org. Chem.* **1991**, *56*,

20   4329, and Delgardo *et. al., Critical Reviews in Therapeutic Drug Carrier Systems* **1992**, *9*, 249. Polyethylene glycols and polyethers include both linear and cyclic compounds. The cyclic polyethers include crown ethers.

As used in this specification the term alkyl includes but is not limited to C_1-C_{12} straight and branched chained alkyls such as methyl, ethyl, propyl, butyl, pentyl, hexyl,

25   heptyl, octyl, nonyl, decyl, undecyl, dodecyl, isopropyl, 2-butyl, isobutyl, 2-methylbutyl, isopentyl, 2-methyl-pentyl, 3-methylpentyl, 2-ethylhexyl and 2-propylpentyl. Alkenyl groups include but are not limited to unsaturated moieties derived from the above alkyl groups including but not limited to vinyl, allyl and crotyl. Alkynyl groups include unsaturated moieties having at least one triple bond that are derived from the above alkyl

30   groups including but are not limited to ethynyl and propargyl. Aryl groups include but are

not limited to phenyl, tolyl, benzyl, naphthyl, anthracyl, phenanthryl, pyrenyl, and xylyl. Halogens include fluorine, chlorine, iodine and bromine. Suitable heterocyclic groups include but are not limited to imidazole, tetrazole, triazole, pyrrolidine, piperidine, piperazine and morpholine. Amines include amines of all of the above alkyl, alkenyl and aryl groups including primary and secondary amines and "masked amines" such as phthalimide. Alkanoyl compounds are those which contain an alkyl portion and are linked through a carbonyl group; i.e., groups which have the structure

alkyl-C(O)-. Aralkyl groups have both aryl and alkyl portions, and are attached through their alkyl portions. A benzyl group is an example of an aralkyl group. Alkaryl groups also have both aryl and alkyl portions, but are attached through their aryl portions. Alkoxy groups are alkyl, alkenyl or alkynyl groups which are attached through an oxygen atom.

Heteroaryl groups are aromatic heretocycles, such as pyrrolyl, furyl, thienyl, imidazolyl, pyridyl, pyrimidinyl, and indolyl.

Terpenes are known in the art as oligomers of isoprene, particularly the dipentenes, pinenes, and myrcenes. Included within the definition of terpene molecules are terpene derivatives such as camphor and menthol.

The term phospholipid as used herein includes those compounds which upon hydrolysis yield phosphoric acid, an alcohol and one or more fatty acids. Representative examples of phospholipids include lecithin, cephalin and sphingomyelin.

Amines are also meant to include polyalkylamino compounds and aminoalkylamines such as aminopropylamine and further heterocyclo-alkylamines such as imidazol-1, 2 or 4-yl-propylamine.

Substituent groups for the above as well as for other moieties listed below include but are not limited to other alkyl, haloalkyl, alkenyl, alkynyl, alkoxy, thioalkoxy, haloalkoxy and aryl groups as well as halogen, hydroxyl, amino, azido, carboxy, cyano, nitro, mercapto, sulfides, sulfones, sulfoxides, keto, carboxy, nitrates, nitrites, nitroso, nitrile, trifluoromethyl, O-alkyl, S-alkyl, NH-alkyl, amino, silyl, amides, ester, ethers, carbonates, carbamates, ureas, imidazoles, intercalators, conjugates, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligonucleotides, and groups that enhance the pharmacokinetic properties of oligonucleo-

tides. Other suitable substituent groups also include rhodamines, coumarins, acridones, pyrenes, stilbenes, oxazolo-pyridocarbazoles, anthraquinones, phenanthridines, phenazines, azidobenzenes, psoralens, porphyrins and cholesterols. One particularly preferred group is CF_3. Typical intercalators and conjugates include cholesterols, phospholipids, biotin, phenanthroline, phenazine, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Halogens include fluorine, chlorine, bromine, and iodine.

One method for preparing compounds according to the invention is to react PNA oligomers or monomeric units bearing at least one free hydroxyl group under basic conditions with a compound having formula L_v-$(CH_2)_n$-S-PG where L_v is a leaving group, PG is a protecting group and n is from 1 to about 20. Displacement of the leaving group through nucleophilic attack of an oxygen anion produces the desired thiol derivative. Suitable leaving groups include but are not limited to halogen, alkylsulfonyl, substituted alkylsulfonyl, arylsulfonyl, substituted arylsulfonyl, hetercyclcosulfonyl, trichloroacetimidate, and pentafluorophenol. A more preferred group includes chloro, fluoro, bromo, iodo, p-(2,4-dinitroanilino)benzenesulfonyl, benzenesulfonyl, methylsulfonyl (mesylate), p-methylbenzenesulfonyl (tosylate), p-bromobenzenesulfonyl, trifluoromethylsulfonyl (triflate), trichloroacetimidate, acyloxy, 2,2,2-trifluoroethanesulfonyl, imidazolesulfonyl, and 2,4,6-trichlorophenyl, with bromo being preferred.

In embodiments wherein thiol-containing functionality is appended to PNA backbone hydroxyl positions, amine functionality in the base portion of the PNA oligomer or monomeric units preferably is protected under non-acidic conditions with protecting groups known in the art, including benzoyl and isobutyryl groups. Alternatively, base protection can precede reaction with thiol reagent L_v-$(CH_2)_n$-S-PG. Suitably protected PNA monomeric units can be assembled into a PNA oligomer according to techniques disclosed in Example 26 of this specification.

Compounds according to the invention can also be prepared by reacting 5-halogen substituted pyrimidine-bearing PNA monomer synthons or 2- or 8-halogen substituted purine PNA monomer synthons with an acetylenic reagent having formula $HC \equiv C$-CH_2-Q_a-PG where Q_a is O, S, or NH, under conditions effective to couple the pyrimidine or purine base with the acetylenic reagent and form a PNA monomer synthon bearing a substituent

having formula $-C\equiv C-CH_2-Q_a-PG$ at the pyrimidine 5-position or at the purine 2- or 8-

position. Numerous suitable protecting groups are known in the art, including, but not

limited to: amine protecting groups such as trifluoroacetate, allyloxycarbonyl (Alloc),

carbobenzyloxy (CBZ), chloroCbz, t-butyloxycarbonyl (Boc), fluorenylmethoxycarbonyl

5   (Fmoc), isonicotinyloxycarbonyl (i-Noc) groups; hydroxyl protecting groups such as *t*-

butyldiphenylsilyl, *t*-butyldimethylsilyl, and dimethoxytrityl groups; and thiol protecting

groups such as S-trityl, S-*p*-methoxybenzylthioether, S-*p*-nitrobenzylthioether, and S-*t*-

butylthioether. (*see, e.g.*, Veber and Hirschmann, *et al., J. Org. Chem.* **1977**, *42*, 3286 and

Atherton, *et al.*, The Peptides, Gross and Meienhofer, Eds, Academic Press; New York,

10   1983; Vol. 9 pp. 1- 38). Coupling preferably is mediated by a metal selected from

palladium, nickel, platinum and iridium under conditions generally in accordance with

Haralambidis, *et al., Nucleic Acids Research* **1987**, *15*, 4857. Once coupling is effected, the

protecting group is removed and the resultant free hydroxy, thio, or amino compound is

condensed with a suitable thiol derivative having formula $R_4-(CH_2)_n-S-R_1$ where R_4 is

15   R_5OOC-, HS, or -NCS where R_5 is H, chloro, alkyl having 1-12 carbon atoms, or an active

ester.

Compounds according to the invention can also be prepared by reacting metal-

substituted pyrimidine bearing PNA monomer synthons or purine bearing PNA monomer

synthons with an acrylate having formula $H_2C=C-C(O)OR_6$ (R_6 = alkyl having 1-12 carbon

20   atoms) under conditions effective to couple the pyrimidine or purine base with the acrylate

and form a PNA monomer synthon bearing a substituent having formula $-CH=CH-C(O)OH$

at the pyrimidine 5-position or at the purine 2- or 8-position. Coupling is effected under

conditions generally in accordance with Dreyer, *et al., Proc. Natl. Acad. Sci. USA* **1985**, *82*,

968. Once coupling is effected, the acid is condensed with an amino thiol derivative having

25   formula $H_2N-(CH_2)_n-S-PG$.

Compounds according to the invention also can be prepared by reacting PNA

monomer synthons bearing leaving groups, L_v as defined above, at 5-pyrimidine positions

or at 2-, 6-, or 8-purine positions with, for example, thiol derivatives having formula HQ_a-

$(CH_2)_n-S-PG$ wherein Q_a and PG are as described above, under conditions effective to

30   displace the leaving group. Such displacement preferably occurs at room temperature in

solvents such as dimethylformamide (DMF) or dimethylsulfoxide (DMSO).

PNA oligomers according to the invention can be prepared by assembling a PNA oligomer and appending thiol functionality thereto. For example, PNA oligomers having free hydroxyl groups can be assembled according to the techniques of Examples **26** and **31-**

5 **37** of this specification, and then reacted with a reagent having formula L_v-$(CH_2)_n$-S-PG. As will be recognized, however, greater selectivity can be achieved in terms of placement of thiol functionality within a PNA oligomer by introducing such functionality, as discussed above, on selected PNA monomer synthons and then using both the selected PNA monomer synthons and other PNA monomer synthons to construct the desired PNA oligomer.

10 Once assembled, a PNA bearing one or more linking moieties ending with -S-PG is treated with a Lewis acid under conditions to remove protecting group PG. Representative acids include silver cation and mercuric cation. Once deprotected, the PNA can be contacted with a thiol-containing steroid molecule, reporter molecule, lipophilic molecule, reporter enzyme, peptide, protein or other thiol containing conjugate in the presence of a

15 thiol-based coupling reagent. Useful coupling reagents include 2,2'-dithiobis(5-nitropyridine) and other pyridyl disulfides or Ellman reagent.

Alternatively, a PNA bearing one or more linking moieties ending with -S-H can be contacted with electrophillic moieties having formula (maleimido)-conjugate or L_v-$CH_2C(O)$-R_2 where L_v is a leaving group as defined above and R_2 is also defined above. As

20 will be recognized, the sulfur atom on the PNA bonds with the former electrophilic moiety via 1,4-addition and with the latter via nucleophilic displacement. Preferred electrophilic moieties include phospholipid maleimide, o-phenanthroline-5-iodoacetamide, fluorescein maleimide, and pyrene maleimide however many alternative electrophilic moieties can be used.

25 Thus, in one aspect of the invention the desired PNA sequence is assembled using standard methods and techniques as set forth in Example **26**. At least one PNA monomer including a masked or protected linking moiety is included in the sequence at a predetermined position. A conjugate is then chemically bonded to the linking moiety directly or via a linking moiety attached to the conjugate.

30 Additional representative thiol groups amenable to the present invention are

cysteine, glutathione, penicillamine, 2-pyridylmercaptyl, Br-CH$_2$-CO-NH-CH$_2$-CH$_2$-STr,

SH-C-(CH$_3$)$_2$CH$_2$-NH-CH$_2$-CH$_2$-NH-CH$_2$-C-(CH$_3$)$_2$SH, HOOC-CH$_2$-CH$_2$-S-S-CH$_3$OOCS,

CH$_3$-CO-S-C(CH$_3$)-CH$_2$-C-NH-(CH$_2$)$_2$-COOH), (see, e.g., Dizio, et al., *Bioconjugate*

Chem. **1991**, *2*, 353 and Greenfield, et al., *Bioconjugate Chem.* **1990**, *1*, 400).

5   Numerous amine protecting groups are known in the art, including, but not limited

to: phthalimide (Phth), trifluoroacetate, allyloxycarbonyl (Alloc), carbobenzyloxy (CBZ),

chloroCBZ, t-butyloxycarbonyl (Boc), fluorenylmethoxycarbonyl (Fmoc), and isonico-

tinyloxycarbonyl (i-Noc) groups. (see, e.g., Veber and Hirschmann, et al., *J. Org. Chem.*

1977, *42*, 3286 and Atherton, et al., The Peptides, Gross and Meienhofer, Eds, Academic

10   Press; New York, 1983; Vol. 9 pp. 1-38).

Conjugate bearing PNA oligomers according to the invention may be prepared by

either functionalizing the PNA monomeric synthons and assembly into the desired

oligomer, or by assembly of PNA monomeric synthons which do not contain conjugate

groups, and subsequently attaching the desired conjugate or conjugates thereto. For

15   example, PNA monomer synthons bearing at least one free hydroxyl group may be reacted

under basic conditions with a compound having formula L$_v$-(CH$_2$)$_n$-NH-PGA where L$_v$ is a

leaving group as defined above and PGA is an amine protecting group. Displacement of the

leaving group through nucleophilic attack of an oxygen anion produces the desired amine

derivative. Suitable leaving groups according to the invention include but are not limited to

20   halogen, alkylsulfonyl, substituted alkylsulfonyl, arylsulfonyl, substituted arylsulfonyl,

hetercyclcosulfonyl or trichloroacetimidate. A more preferred group includes chloro,

fluoro, bromo, iodo, p-(2,4-dinitroanilino)benzenesulfonyl, benzenesulfonyl, methylsulfonyl

(mesylate), p-methylbenzenesulfonyl (tosylate), p-bromobenzenesulfonyl, trifluoromethyl-

sulfonyl   (triflate),   trichloroacetimidate,   acyloxy,   2,2,2-trifluoroethanesulfonyl,

25   imidazolesulfonyl, and 2,4,6-trichlorophenyl, with bromo being preferred.

PNA oligomers according to the invention can also be prepared by assembling a

PNA oligomer and appending alkylamino functionality thereto. For example, PNA

oligomers having free hydroxyl groups can be assembled according to known techniques

see Examples **26** and **31-37** and then reacted with a reagent having formula L$_v$-(CH$_2$)$_n$-NH-

30   PGA. As will be recognized, however, greater selectivity can be achieved in terms of

placement of alkylamino functionality within a PNA oligomer by introducing such functionality, as discussed above, on selected PNA monomeric synthons and then using both the selected PNA monomeric synthons and other PNA monomeric synthons to construct a desired PNA oligomer.

5   Once assembled, a PNA bearing one or more groups having a linking moiety ending with a protected amine group is treated with reagents effective to remove the protecting group. Once deprotected, the PNA can be contacted with electrophilic moieties such as, for example, succinimidyl esters and other activated carboxylic acids including C(=O)-O-succinimide and C(=O)-O-pentafluorophenyl, isothiocyanates, sulfonyl chlorides,

10   haloacetamides, phospholipid carbocyclic acid active esters, o-phenanthroline-5-iodoacetamide, fluorescein isothiocyanate, 1-pyrene butyric acid-N-hydroxy succinimide ester and carboxylic acid derivatives of peptide nucleic acids. Preferred electrophilic moieties include cholesteryl-3-hemisuccinate-N-hydroxy succinimide ester, pyrene-1-butyric acid-N-hydroxy succinimide ester and polyethylene glycol-propionic acid-N-

15   hydroxy succimide ester.

         In other aspects of the invention a conjugate is attached to at least one monomeric unit of a PNA in a diagnostic or therapeutic agent to assist in the transfer of therapeutic or diagnostic agent across cellular membranes. Such a diagnostic or therapeutic agent is formed from a plurality of linked PNA monomeric units bearing natural or non-natural

20   occurring bases and including at least one conjugate which, can include an optional linking moiety, appended thereto, in a sequence that is complementary to and will specifically hybridize with a region of an RNA or DNA of interest. Thus, one or more of the linked PNA monomeric units are "functionalized" to include a conjugate linked to the PNA monomeric unit. This linkage may include an optional linking moiety. Linked PNA

25   monomer units having at least one functionalized PNA monomer unit within their sequence are expected to demonstrate enhanced activity and transport across cellular membranes.

         A variety of linking groups can be used to connect conjugates to PNA oligomers or monomer synthons. Certain linking groups, such as Ω-aminoalkoxy moieties and Ω-aminoalkylamino moieties, are particularly useful for linking steroid molecules or reporter

30   molecules to PNA hydroxyl groups. Many linking groups are commercially available,

including heterobifunctional and homobifunctional linking moieties available from the Pierce Co. (Rockford, Il). Heterobifunctional and homobifunctional linking moieties are particularly useful in conjunction with the Ω-aminoalkoxy and Ω-aminoalkylamino moieties to form extended linkers that connect peptides and proteins to PNA oligomers. It is intended that the PNA monomer synthons of the invention include adenine bearing PNA synthons functionalized with linkers on their N-6 purine amino groups, guanine bearing PNA monomer synthons functionalized with linkers at their exocyclic N-2 purine amino groups, and cytosine bearing PNA monomer synthons functionalized with linkers on either their N-4 pyrimidine amino groups or 5 pyrimidine positions. This type of linkage is illustrated by the attachment of the conjugate biotin in a PNA 5-mer to the N-4 position of cytosine in Example **47**.

A PNA oligomer of the invention may be reacted with an active ester derivative of a conjugate (*e.g.*, cholic acid). Active ester derivatives include N-hydroxysuccinimide esters, tetrafluorophenolic esters, pentafluorophenolic esters and pentachlorophenolic esters. For cholic acid, the reaction of the amino group and the active ester produces a PNA oligomer in which cholic acid is attached to the N-terminal position through a linking group. Cholic acid can be attached to the carboxy terminal of a PNA oligomer by conversion of the cholic acid to the N-hydroxysuccinimide ester thereof, and then by further reaction with the PNA of interest. Cholic acid can be attached to both ends of a PNA oligomer by applying both methods above. As will be seen by those skilled in the art, the above methods are applicable to a large number of conjugates of the present invention.

In further embodiments of the invention, a PNA oligomer bearing an aminolinker at one or more selected non-terminal monomeric units is prepared according to the procedures of Examples **26** and **65**. A conjugate such as cholic acid is then attached using an active ester or an isothiocyanate thereof. This approach allows the introduction of a large number of conjugates into a single PNA oligomeric sequence. Indeed each of the PNA monomers in the oligomer can be so substituted.

Attachment of conjugates such as reporter enzymes, peptides, and proteins to PNAs is achieved by reaction of the enzyme, peptide or protein with the above-described amino linking group on the PNA by any of several methods. For example, a peptide or protein can

be attached to a functionalized PNA monomer by treatment of the peptide or protein with EDC/sulfo-NHS (*i.e.*, 1-ethyl-3(3-dimethylaminopropylcarbodiimide/N-hydroxysulfo-succinimide), which will facilitate linkage of the carboxyl end of the reporter enzyme, peptide, or protein to the amino terminal end of the PNA, or to an amine-containing linker bound to the PNA. Further, a PNA oligomeric conjugate can be prepared using EDC/sulfo-NHS to attach a carboxyl group of an aspartic or glutamic acid residue in the reporter enzyme, peptide or protein to the amino terminus of a PNA.

Preferably a reporter enzyme-, peptide- or protein-functionalized PNA can be prepared by treating the reporter enzyme, peptide or protein with the PNA oligomer functionalized with a heterobifunctional linker such as *m*-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (MBS) or succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC). Thus a thiol function on the reporter enzyme, peptide or protein is linked to the amino function of the PNA. A PNA oligomer-maleimide conjugate is formed by reaction of the amino group of the PNA-bound linker with the MBS or SMCC maleimide linker. The resulting conjugate is then reacted with peptides or proteins having free sulfhydryl groups.

In a second preferred method, a reporter enzyme-, peptide- or protein-functionalized linked PNA oligomer can be prepared by treatment of the peptide or protein with the PNA using a homobifunctional linker such as disuccinimidyl suberate (DSS) to link an amino function on the peptide or protein to the amino group of a linker on the PNA. By this mechanism, a succinimidyl functionality can be attached to a PNA oligomer by reaction of the amino group of the PNA-bound linker with a disuccinimidyl suberate linker. The disuccinimidyl suberate linker couples with the amine linker on the sequence to extend the size of the linker. The extended linker is then reacted with amine groups such as, for example, the amine of lysine or other available N-terminus amines on reporter enzymes, peptides and proteins.

PNA oligomers of the present invention may range in size from a dimer to a 200-mer. In preferred embodiments of the present invention the PNA oligomers range in size from a dimer to a 50-mer. In more preferred embodiments the PNA oligomers range in size from a dimer to about a 20-mer.

In further embodiments a 13- to 14-mer PNA is used following standard methods and techniques to identify a region of a DNA coding for a message that is present for the expression of a specific mRNA.

Although longer PNA molecules are envisioned by the present invention it can readily be seen that PNAs having from 1 to about 20 monomeric units will be useful for a number of diagnostic applications.

The compounds of the present invention also will be useful as research reagents for the modulation of the production of a protein by an organism. Modulation may be accomplished by contacting the organism with compounds of the present invention. Preferably the compounds are hybridizable with RNA/DNA coding for the protein of interest.

The compounds of the present invention will additionally be useful as diagnostic reagents. Diagnostic applications include the detection of the presence or absence of a particular DNA or RNA in a sample. The sample of interest is contacted with a compound of the invention wherein the compound is specifically hybridizable with the RNA of interest. Methods of detecting the hybridization of the diagnostic reagent to the DNA or RNA of interest or alternatively the absence of such hybridization include but are not limited to detection by fluorescence, radiolabeling or capillary gel electrophoresis. PNAs of the present invention are labeled by attachment of the appropriate conjugate thereto. The sample of interest is contacted with the PNA having at least one conjugate attached thereto. The determination of the presence or absence of the nucleic acid of interest will be facilitated by the detection of the presence or absence of hybridization to the sample.

In a further aspect of the invention, the PNAs are conjugated to peptides or proteins, where the peptides have signaling activity and the proteins are, for example, enzymes, transcription factors or antibodies. Also, the PNAs can be attached to water-soluble or water-insoluble polymers.

In another aspect of the invention, the PNAs are conjugated to oligonucleotides or carbohydrates. When desired, a PNA oligomer can be synthesized onto a moiety (*e.g.*, a peptide chain, reporter, intercalator or other type of ligand-containing group) which is attached to a solid support.

As a further aspect of the present invention, PNAs can be used to target RNA and ssDNA to produce hybridization probes for the identification and purification of nucleic acids. Furthermore, the PNAs can be modified to form strand displacement triple helices with dsDNA. Thus, these reagents may be used for research and in diagnostics for detection

5 and isolation of specific nucleic acids.

Triple helix formation, wherein an oligonucleotide is triplexed to a dsDNA, is believed to be the only means known in the art for sequence-specific recognition of dsDNA. However, triple helix formation is largely limited to recognition of homopurine-homopyrimidine sequences. Triplexing with strand displacement using PNAs of this

10 invention is superior to oligonucleotide-dsDNA triple helix recognition in that it may allow for recognition of any sequence by use of the four natural bases.

PNAs of the present invention may be useful as research and diagnostic reagents for the detection of a gene. PNAs are designed with a nucleobase sequence containing from about 12 to about 18 sub-units that are complementary to the regulatory region (the

15 promoter) of the target gene. Samples (e.g. cells, tissue, or other) of interest are prepared following standard methods and techniques and are treated in vitro with a PNA (in a suitable carrier) of the present invention having a specific sequence and at least one conjugate attached to at least one sub-unit. The PNA will bind to the promoter and block access thereto by RNA polymerase. Consequently, no mRNA, and thus no gene product

20 (protein), is produced. If the target is within a vital gene for a virus, no viable virus particles will be produced. Alternatively, if the target is downstream from the promoter, RNA polymerase will terminate at this position, thus forming a truncated mRNA/protein which is nonfunctional.

Sequence-specific recognition of ssDNA by base complementary hybridization can

25 be exploited to target specific genes and viruses. In this case, the target sequence is contained in the mRNA such that binding of the drug to the target hinders the action of ribosomes and, consequently, translation of the mRNA into protein. The peptide nucleic acids of the invention are superior to prior reagents in that they have significantly higher affinity for complementary ssDNA. Additionally, because PNAs do not possess a charged

30 backbone, lysine or other similar charged moieties can be present without interfering with

PNA activity. Additionally, PNAs are water soluble (which should facilitate cellular uptake), and contain amides of non-biological amino acids (which should make them biostable and resistant to enzymatic degradation by, for example, proteases), and are capable of triplex formation with mRNA.

5   Binding of PNA compounds to double-stranded DNA accompanied by strand displacement is shown in United States application serial number 08/088,658, filed July 2, 1993, entitled "Higher Order Structure and Binding of Peptide Nucleic Acids". Binding of PNA and bis PNA to double stranded DNA accompanied by strand invasion is also shown in United States application serial number 08/275,951, filed July 15, 1994, entitled "Linked

10   Peptide Nucleic Acids". The illustrative examples therein show that PNA compounds bind via Watson-Crick binding to their complementary strand and extrudes the other strand in a virtually single-stranded conformation.

The chimeric structure between PNAs and DNA or RNA are used in place of, or in addition to a normal PNA strand to effect duplexing, triplexing, nucleic acid binding or

15   protein binding. The RNA or DNA nucleic acid portion of such chimeric structures include but are not limited to a nucleic acid connected via phosphodiester, phosphorothioate, phosphorodithioate, alkyl phosphonate, hydrogen phosphonate, phosphotriester, phosphoramidite and other like phosphorus linkages. They further can include but are not limited to other substitutions such as substitution at the 2' position of a ribose sugar.

20   Particularly preferred are 2'-fluoro's since they increase affinity of the nucleic acid portion of the chimera to other nucleic acids and 2'-O-alkyl, particularly 2'-O-propyl, 2'-O-allyl and the like since they confer nuclease resistance to the nucleic acid strand. The compounds of the present invention may be useful to bind to other target molecules. Target molecules of the present invention can include any of a variety of biologically significant molecules.

25   Such other target molecules can be carbohydrates, glycoproteins or other proteins. In some preferred embodiments of the present invention, the target molecule is a protein such as an immunoglobulin, receptor, receptor binding ligand, antigen or enzyme and more specifically can be a phospholipase, tumor necrosis factor, endotoxin, interleukin, plasminogen activator, protein kinase, cell adhesion molecule, lipoxygenase, hydrolase or transacylase. In

30   other embodiments of the invention the target molecules can be important regions of the

human immunodeficiency virus, *Candida*, herpes viruses, papillomaviruses, cytomegalovirus, rhinoviruses, hepatitis viruses, or influenza viruses. In yet other embodiments of the present invention the target molecules can be regions of an oncogene. In still further embodiments, the target molecule is ras 47-mer stem loop RNA, the TAR

5   element of human immunodeficiency virus, rev response element (RRE) or the gag-pol stem loop of human immunodeficiency virus (HIV). Still other targets can induce cellular activity. For example, a target can induce interferon.

In binding to transcription factors or other target molecules, the transcription factor or other target molecule need not be purified. It can be present, for example, in a whole

10   cell, in a humoral fluid, in a crude cell lysate, in serum or in other humoral or cellular extract. Of course, purified transcription factor or a purified form of another target molecule is also useful in some aspects of the invention.

In still other embodiments of the present invention, synthetically prepared transcription factor or other target molecules can be useful. These synthetically prepared

15   molecules need only contain at least one monomeric unit having a conjugate attached thereto. A transcription factor or other target molecule also can be modified, such as by biotinylation or radiolabeling. For example, synthetically prepared transcription factor can incorporate one or more biotin molecules during synthesis or can be modified post-synthesis.

20   Transcription factors, as the term is used herein, are DNA- or RNA-binding proteins that regulate the expression of genes. HIV tat and c-rel are examples of transcription factors which regulate the expression of genes. Also encompassed by the term are DNA and RNA binding proteins which are not strictly considered transcription factors, but which are known to be involved in cell proliferation. These transcription factors include c-myc, fos,

25   and jun. Methods of the present invention are particularly suitable for use with transcription factor as target molecules since transcription factors generally occur in very small cellular quantities.

The utility of PNA-containing duplex structures can be illustrated by constructing PNA sequences which correspond to various sequences of the HIV TAR element that have

30   the potential to form duplex structures either as stem-loop structures or two PNAs forming a

duplex structure. In a competition assay, PNA structures that bind the tat transcription factor prevent binding of the competitor TAR sequence present in the incubation mixture. As the TAR RNA sequence is biotinylated only that proteins available to bind to TAR will remain on the microtiter plate after washing away unbound molecules and tat protein complexed to a PNA sequence. The concentration dependence of the competition between the TAR PNA structures and biotinylated TAR structure will serve to define those sequences capable of effectively competing for tat and thus useful as HIV modulatory agents.

Additional objects, advantages, and novel features of this invention will become apparent to those skilled in the art upon examination of the following examples thereof, which are not intended to be limiting.

EXAMPLE 1

tert-Butyl 4-nitrophenyl carbonate (1)

Sodium carbonate (29.14 g; 0.275 mol) and 4-nitrophenol (12.75 g; 91.6 mmol) were mixed with dioxane (250 ml). Boc-anhydride (20.0 g; 91.6 mmol) was transferred to the mixture with dioxane (50 ml). The mixture was refluxed for 1 h, cooled to 0°C, filtered and concentrated to 1/3, and then poured into water (350 ml) at 0°C. After stirring for 1/2 h., the product was collected by filtration, washed with water, and then dried over an appropriate drying agent, *in vacuo*. Yield 21.3 g (97%). M.p. 73.0-74.5°C (litt. 78.5-79.5°C). Anal. for $C_{11}H_{13}NO_5$ found(calc.) C: 55.20(55.23) H: 5.61(5.48) N: 5.82(5.85).

EXAMPLE 2

(N-Boc-2-aminoethyl)glycine (2)

The title compound was prepared by a modification of the procedure by Heimer, *et al. Int. J. Pept.*, **1984**, *23*, 203-211 N-(2-Aminoethyl)glycine (3.00 g; 25.4 mmol) was dissolved in water (50 ml), dioxane (50 ml) was added, and the pH was adjusted to 11.2 with 2 N sodium hydroxide. *tert*-Butyl-4-nitrophenyl carbonate (1, 7.29 g; 30.5 mmol) was dissolved in dioxane (40 ml) and added dropwise over a period of 2 h, during which time the pH was maintained at 11.2 with 2 N sodium hydroxide. The pH was adjusted

periodically to 11.2 for three more hours and then the solution was left overnight. The solution was cooled to 0°C and the pH was carefully adjusted to 3.5 with 0.5 M hydrochloric acid. The aqueous solution was washed with chloroform (3 x 200 ml), the pH adjusted to 9.5 with 2N sodium hydroxide and the solution was evaporated to dryness, *in vacuo* (14 mm Hg). The residue was extracted with DMF (25+2x10 ml) and the extracts filtered to remove excess salt. This results in a solution of the title compound in about 60% yield and greater than 95% purity by tlc (visualized by ninhydrin, Rf=0.3). The solution was used in the following preparations of Boc-aeg (aeg=aminoethylglycine) derivatives without further purification.

EXAMPLE 3

N-1-Carboxymethylthymine (3)

This procedure is different from the literature synthesis, but is easier, gives higher yields, and leaves no unreacted thymine in the product. To a suspension of thymine (40.0 g; 0.317 mol) and potassium carbonate (87.7 g; 0.634 mmol) in DMF (900 ml) was added methyl bromoacetate (30.00 ml; 0.317 mmol). The mixture was stirred vigorously overnight under nitrogen. The mixture was filtered and evaporated to dryness, *in vacuo*. The solid residue was treated with water (300 ml) and 4 N hydrochloric acid (12 ml), stirred for 15 min at 0°C, filtered, and washed with water (2 x 75 ml). The precipitate was treated with water (120 ml) and 2N sodium hydroxide (60 ml), and was boiled for 10 minutes. The mixture was cooled to 0°C, filtered, and the pure title compound was precipitated by the addition of 4 N hydrochloric acid (70 ml). Yield after drying, *in vacuo* over an appropriate drying agent: 37.1 g (64%). ^{1}H-NMR: (90 MHz; DMSO-d$_6$): 11.33 ppm (s,1H,N$\underline{\text{H}}$); 7.49(d,J=0.92Hz,1H,Ar$\underline{\text{H}}$); 4.38 (s,2H,C$\underline{\text{H}}_2$); 1.76 (d,J=0.92Hz,T-C$\underline{\text{H}}_3$).

EXAMPLE 4

N-1-Carboxymethylthymine pentafluorophenyl ester (4)

N-1-Carboxymethylthymine (3, 10.0g; 54.3 mmol) and pentafluorophenol (10.0 g; 54.3 mmol) were dissolved in DMF (100 ml) and cooled to 5°C in ice water. DCC (13.45 g; 65.2 mmol) then was added. When the temperature passed below 5°C, the ice bath was

removed and the mixture was stirred for 3 h at ambient temperature. The precipitated DCU was removed by filtration and washed twice with DMF (2 x 10 ml). The combined filtrate was poured into ether (1400 ml) and cooled to 0°C. Petroleum ether (1400 ml) was added and the mixture was left overnight. The title compound was isolated by filtration and was

5 washed thoroughly with petroleum ether. Yield: 14.8 g(78%). The product was pure enough to carry out the next reaction, but an analytical sample was obtained by recrystallization from 2-propanol. M.p. 200.5-206 °C Anal. for $C_{13}H_7F_5N_2O_4$. Found(calc.) C: 44.79(44.59); H: 2.14(2.01) N: 8.13(8.00). FAB-MS: 443 (M+1+glycerol), 351 (M+1). ^{1}H-NMR (90 MHz; DMSO-d_6): 11.52 ppm (s,1H,N$\underline{H}$); 7.64 (s,1H,Ar$\underline{H}$); 4.99 (s,2H,C$\underline{H}_2$);

10 1.76 (s,3H,C$\underline{H}_3$).

EXAMPLE 5

N-(N-Boc-2-aminoethyl)-N-[(1-thyminyl)acetyl]glycine (5)

To the DMF-solution from Example **2** was added triethyl amine (7.08 ml; 50.8

15 mmol) followed by N-1-carboxymethylthymine pentafluorophenyl ester (**4**, 4.45 g; 12.7 mmol). The resultant solution was stirred for 1 h. The solution was cooled to 0°C and treated with cation exchange material ("Dowex 50W X-8", 40 g) for 20 min. The cation exchange material was removed by filtration, washed with dichloromethane (2 x 15 ml), and dichloromethane (150 ml) was added. The resulting solution was washed with

20 saturated sodium chloride, dried over magnesium sulfate, and evaporated to dryness, *in vacuo*, first by a water aspirator and then by an oil pump. The residue was shaken with water (50 ml) and evaporated to dryness. This procedure was repeated once. The residue then was dissolved in methanol (75 ml) and poured into ether (600 ml) and petroleum ether (1.4 L). After stirring overnight, the white solid was isolated by filtration and was washed

25 with petroleum ether. Drying over an appropriate drying agent, *in vacuo*, gave 3.50 g (71.7%). M.p. 142-147°C. Anal. for $C_{16}H_{24}N_4O_7$. Found(calc.) C: 49.59(50.00) H: 6.34(6.29) N: 14.58(14.58). ^{1}H-NMR (250 MHz, DMSO-d_6): Due to the limited rotation around the secondary amide bond several of the signals were doubled in the ratio 2:1,(indicated in the list by mj. for major and mi. for minor). 12.73 ppm (b,1H, -CO$_2$H);

30 11.27 ppm (s, mj., imide); 11.25 ppm (s, mi., imide); 7.30 ppm (s, mj., ArH); 7.26 ppm (s,

mi., ArH); 6.92 ppm (unres. t, mj., BocNH); 6.73 ppm (unres. t; mi., BocNH); 4.64 ppm (s, mj., T-CH$_2$-CO-); 4.47 ppm (s, mi., T-CH$_2$-CO-); 4.19 ppm (s, mi., CONRC$\underline{H}_2$CO$_2$H); 3.97 ppm (s, mj., CONRC$\underline{H}_2$CO$_2$H); 3.41-2.89 ppm (unres. m, -CH$_2$CH$_2$- and water); 1.75 ppm (s,3H, T-CH$_3$); 1.38 ppm (s, 9H, t-Bu). ^{13}C-NMR: 170.68 ppm (CO); 170.34 (CO); 167.47 (CO); 167.08 (CO); 164.29 (CO); 150.9 (C5"); 141.92 (C6"); 108.04 (C2'); 77.95 and 77.68 (Thy-C$\underline{H}_2$CO); 48.96, 47.45 and 46.70 (-$\underline{C}$H$_2$C$\underline{H}_2$- and NC$\underline{H}_2$CO$_2$H); 37.98 (Thy-C$\underline{H}_3$); 28.07 (t-Bu). FAB-MS: 407 (M+Na$^+$); 385 (M+H$^+$).

EXAMPLE 6

N-(N-Boc-2-aminoethyl)-N-[(1-thyminyl)acetyl]glycine pentafluorophenyl ester (6, Boc-Taeg.OPfp)

N-(N-Boc-2-aminoethyl)-N-[(1-thyminyl)acetyl]glycine (5) (2.00 g; 5.20 mmol) was dissolved in DMF (5 ml) and methylene chloride (15 ml) was added. Pentafluorophenol (1.05 g; 5.72 mmol) was added and the solution was cooled to 0°C in an ice bath. DDC then was added (1.29 g; 6.24 mmol) and the ice bath was removed after 2 min. After 3 h with stirring at ambient temperature, the precipitated DCU was removed by filtration and washed with methylene chloride. The combined filtrate was washed twice with aqueous sodium hydrogen carbonate and once with saturated sodium chloride, dried over magnesium sulfate, and evaporated to dryness, *in vacuo*. The solid residue was dissolved in dioxane (150 ml) and poured into water (200 ml) at 0°C. The title compound was isolated by filtration, washed with water, and dried over an appropriate drying agent, *in vacuo*. Yield: 2.20 g (77%). An analytical sample was obtained by recrystallisation from 2-propanol. M.p. 174-175.5°C. Analysis for C$_{22}$H$_{23}$N$_4$O$_7$F$_5$, found(calc.): C: 48.22(48.01); H: 4.64(4.21); N: 9.67(10.18). ^{1}H-NMR (250 MHz, CDCl$_3$):Due to the limited rotation around the secondary amide bond several of the signals were doubled in the ratio 6:1 (indicated in the list by mj. for major and mi. for minor). 7.01 ppm (s, mi., ArH); 6.99 ppm (s, mj., ArH); 5.27 ppm (unres. t, BocN$\underline{H}$); 4.67 ppm (s, mj., T-CH$_2$-CO-); 4.60 ppm (s, mi., T-CH$_2$-CO-); 4.45 ppm (s, mj., CONRC$\underline{H}_2$CO$_2$Pfp); 4.42 ppm (s, mi., CONRC$\underline{H}_2$CO$_2$Pfp); 3.64 ppm (t,2H,BocNHCH$_2$C$\underline{H}_2$-); 3.87 ppm ("q",2H,BocNHC$\underline{H}_2$CH$_2$-); 1.44(s,9H,t-Bu). FAB-MS: 551 (10; M+1); 495 (10; M+1-tBu); 451 (80; -Boc).

EXAMPLE 7

N-(N-Boc-2-aminoethyl)-N-(Cbz)glycine (7)

Aminoethyl glycine (52.86 g; 0.447 mol) was dissolved in water (900 ml) and
5 dioxane (900 ml) was added. The pH was adjusted to 11.2 with 2N NaOH. While the pH
was kept at 11.2, tert-butyl-p-nitrophenyl carbonate (128.4 g; 0.537 mol) was dissolved in
dioxane (720 ml) and added dropwise over the course of 2 hours. The pH was kept at 11.2
for at least three more hours and then left with stirring overnight. The yellow solution was
cooled to 0°C and the pH was adjusted to 3.5 with 2 N HCl. The mixture was washed with
10 chloroform (4x100 ml), and the pH of the aqueous phase was readjusted to 9.5 with 2 N
NaOH at 0°C. Cbz chloride (73.5 ml; 0.515 mol) was added over half an hour, while the
pH was kept at 9.5 with 2 N NaOH. The pH was adjusted frequently over the next 4 hours,
and the solution was left with stirring overnight. On the following day the solution was
washed with ether (3x600 ml) and the pH of the solution was afterwards adjusted to 1.5
15 with 2 N HCl at 0°C. The title compound was isolated by extraction with ethyl acetate
(5x1000 ml). The ethyl acetate solution was dried over magnesium sulfate and evaporated
to dryness, in vacuo. This afforded 138 g, which was dissolved in ether (300 ml) and
precipitated by the addition of petroleum ether (1800 ml). Yield 124.7 g (79%). M.p. 64.5-
85 °C. Anal. for $C_{17}H_{24}N_2O_6$ found(calc.) C: 58.40(57.94); H: 7.02(6.86); N: 7.94(7.95).
20 ^{1}H-NMR (250 MHz, CDCl$_3$) 7.33 & 7.32 (5H, Ph); 5.15 & 5.12 (2H, PhC$\underline{H}_2$); 4.03 & 4.01
(2H, NC$\underline{H}_2$CO$_2$H); 3.46 (b, 2H, BocNHCH$_2$C$\underline{H}_2$); 3.28 (b, 2H, BocNHC$\underline{H}_2$CH$_2$); 1.43 &
1.40 (9H, ^{t}Bu). HPLC (260 nm) 20.71 min. (80.2%) and 21.57 min. (19.8%). The UV-
spectra (200 nm - 300 nm) are identical, indicating that the minor peak consists of Bis-Z-
AEG.

25

EXAMPLE 8

N-(N-Boc-2-aminoethyl)glycine ethyl ester (8)

N-(N-Boc-2-aminoethyl)-N-(Cbz)glycine (7, 60.0 g; 0.170 mol) and N,N-dimethyl-
4-aminopyridine (6.00 g) were dissolved in absolute ethanol (500 ml), and cooled to 0°C
30 before the addition of DCC (42.2 g; 0.204 mol). The ice bath was removed after 5 minutes

and stirring was continued for 2 more hours. The precipitated DCU (32.5 g dried) was removed by filtration and washed with ether (3x100 ml). The combined filtrate was washed successively with diluted potassium hydrogen sulfate (2x400 ml), diluted sodium hydrogencarbonate (2x400 ml) and saturated sodium chloride (1x400 ml). The organic phase was filtered, then dried over magnesium sulfate, and evaporated to dryness, *in vacuo*, which yielded 66.1 g of an oily substance which contained some DCU.

The oil was dissolved in absolute ethanol (600 ml) and was added 10% palladium on carbon (6.6 g) was added. The solution was hydrogenated at atmospheric pressure, where the reservoir was filled with 2 N sodium hydroxide. After 4 hours, 3.3 L was consumed out of the theoretical 4.2 L. The reaction mixture was filtered through celite and evaporated to dryness, *in vacuo*, affording 39.5 g (94%) of an oily substance. A 13 g portion of the oily substance was purified by silica gel (600 g SiO$_2$) chromatography. After elution with 300 ml 20% petroleum ether in methylene chloride, the title compound was eluted with 1700 ml of 5% methanol in methylene chloride. The solvent was removed from the fractions with satisfactory purity, *in vacuo* and the yield was 8.49 g. Alternatively 10 g of the crude material was purified by Kugel Rohr distillation. ^{1}H-NMR (250 MHz, CD$_3$OD); 4.77 (b. s, NH); 4.18 (q, 2H, MeC$\underline{H}_2$-); 3.38 (s, 2H, NC$\underline{H}_2$CO$_2$Et); 3.16 (t, 2H, BocNHC$\underline{H}_2$CH$_2$); 2.68 (t, 2H, BocNHCH$_2$C$\underline{H}_2$); 1.43 (s, 9H, [t]Bu) and 1.26 (t, 3H, CH$_3$) ^{13}C-NMR 171.4 ($\underline{C}$OEt); 156.6 (CO); 78.3 ((CH$_3$)$_3$$\underline{C}$); 59.9 (CH$_2$); 49.0 (CH$_2$); 48.1 (CH$_2$); 39.0 (CH$_2$); 26.9 (CH$_2$) and 12.6 (CH$_3$).

EXAMPLE 9

N-(N-Boc-2-aminoethyl)glycine methyl ester (9)

The procedure of Example 8 was used, with methanol being substituted for ethanol. The final product was purified by flash column chromatography.

EXAMPLE 10

N-(N-Boc-2-aminoethyl)-N-[(1-thyminyl)acetyl]glycine ethyl ester (10)

N-(N-Boc-2-aminoethyl)glycine ethyl ester (8, 13.5 g; 54.8 mmol), DhbtOH (9.84 g; 60.3 mmol) and N-1-carboxymethyl thymine (4, 11.1 g; 60.3 mmol) were dissolved in

DMF (210 ml). Methylene chloride (210 ml) then was added. The solution was cooled to 0°C in an ethanol/ice bath and DCC (13.6 g; 65.8 mmol) was added. The ice bath was removed after 1 hour and stirring was continued for another 2 hours at ambient temperature. The precipitated DCU was removed by filtration and washed twice with methylene chloride

5   (2 x 75 ml). To the combined filtrate was added more methylene chloride (650 ml). The solution was washed successively with diluted sodium hydrogen carbonate (3 x 500 ml), diluted potassium hydrogen sulfate (2 x 500 ml), and saturated sodium chloride (1 x 500 ml). Some precipitate was removed from the organic phase by filtration, The organic phase was dried over magnesium sulfate and evaporated to dryness, *in vacuo*. The oily residue

10   was dissolved in methylene chloride (150 ml), filtered, and the title compound was precipitated by the addition of petroleum ether (350 ml) at 0°C. The methylene chloride/petroleum ether procedure was repeated once. This afforded 16.0 g (71%) of the title compound which was more than 99% pure by HPLC.

15   **EXAMPLE 11**

N-(N-Boc-2-aminoethyl)-N-[(1-thyminyl)acetyl]glycine (6a)

The material from Example **10** was suspended in THF (194 ml, gives a 0.2 M solution), and 1 M aqueous lithium hydroxide (116 ml) was added. The mixture was stirred for 45 minutes at ambient temperature and then filtered to remove residual DCU. Water (40

20   ml) was added to the solution which was then washed with methylene chloride (300 ml). Additional water (30 ml) was added, and the alkaline solution was washed once more with methylene chloride (150 ml). The aqueous solution was cooled to 0°C and the pH was adjusted to 2 by the dropwise addition of 1 N HCl (approx. 110 ml). The title compound was extracted with ethyl acetate (9 x 200 ml), the combined extracts +were dried over

25   magnesium sulfate and were evaporated to dryness, *in vacuo*. The residue was evaporated once from methanol, which after drying overnight afforded a colorless glassy solid. Yield 9.57 g (64 %). HPLC > 98% R_T=14.8 min . Anal. for $C_{16}H_{24}N_4O_7$/0.25 H_2O Found (calc.) C: 49.29(49.42); H: 6.52(6.35); N: 14.11(14.41). Due to the limited rotation around the secondary amide, several of the signals were doubled in the ratio 2:1 (indicated in the list by

30   mj. for major and mi. for minor). ^{1}H-NMR (250 MHz, DMSO-d_6): 12.75 (b.s., 1H, CO_2H);

11.28 (s, "1H", mj., imide NH); 11.26 (s, "1H", mi., imide NH); 7.30 (s, "1H", mj., T H-6); 7.26 (s, "1H", mi., T H-6); 6.92 (b.t., "1H", mj., BocNH); 6.73 (b.t., "1H", mi., BocNH); 4.64 (s, "2H", mj., C$\underline{H}_2$CON); 4.46 (s, "2H", mj., C$\underline{H}_2$CON); 4.19 (s, "2H", mi., C$\underline{H}_2$CO$_2$H); 3.97 (s, "2H", mj., C$\underline{H}_2$CO$_2$H); 3.63-3.01 (unresolved m, includes water,

5   C$\underline{H}_2$C$\underline{H}_2$); 1.75 (s, 3H, C$\underline{H}_3$) and 1.38 (s, 9H, ^{t}Bu).

EXAMPLE 12

N-4-Cbz cytosine (12)

Over a period of about 1 h, Cbz chloride (52 ml; 0.36 mol) was added dropwise to a

10   suspension of cytosine (8, 20.0 g;0.18 mol) in dry pyridine (1000 ml) at 0°C under nitrogen in oven-dried equipment. The solution then was stirred overnight, after which the pyridine suspension was evaporated to dryness, in vacuo. Water (200 ml) and 4 N hydrochloric acid were added to reach pH ~1. The resulting white precipitate was filtered off, washed with water and partially dried by air suction. The still-wet precipitate was boiled with absolute

15   ethanol (500 ml) for 10 min, cooled to 0°C, filtered, washed thoroughly with ether, and dried, in vacuo. Yield 24.7 g (54%). M.p.>250°C. Anal. for C12H11N3O3. Found(calc.); C: 58.59(58.77); H: 4.55(4.52); N: 17.17(17.13). No NMR spectra were recorded since it was not possible to get the product dissolved.

20   ## EXAMPLE 13

N-4-Cbz-N-1-carboxymethyl cytosine (13)

In a three necked round bottomed flask equipped with mechanical stirring and nitrogen coverage was placed methyl bromacetate (7.82 ml;82.6 mmol) and a suspension of N-4-Cbz cytosine (12, 21.0 g;82.6 mmol) and potassium carbonate (11.4 g;82.6 mmol) in

25   dry DMF (900 ml). The mixture was stirred vigorously overnight, filtered, and evaporated to dryness, *in vacuo*. Water (300 ml) and 4 N hydrochloric acid (10 ml) were added, the mixture was stirred for 15 minutes at 0°C, filtered, and washed with water (2 x 75 ml). The isolated precipitate was treated with water (120 ml), 2N sodium hydroxide (60 ml), stirred for 30 min, filtered, cooled to 0°C, and 4 N hydrochloric acid (35 ml) was added. The title

30   compound was isolated by filtration, washed thoroughly with water, recrystallized from

methanol (1000 ml) and washed thoroughly with ether. This afforded 7.70 g (31%) of pure

compound. The mother liquor from the recrystallization was reduced to a volume of 200 ml

and cooled to 0°C. This afforded an additional 2.30 g of a material that was pure by tlc but

had a reddish color.   M.p. 266-274°C.   Anal. for $C_{14}H_{13}N_3O_5$. Found(calc.); C:

5   55.41(55.45); H: 4.23(4.32); N: 14.04(13.86). ^{1}H-NMR (90 MHz; DMSO-d_6): 8.02 ppm

(d,J=7.32Hz,1H,H-6); 7.39 (s,5H,Ph); 7.01 (d,J=7.32Hz,1H,H-5); 5.19 (s,2H,PhC$\underline{H}_2$-); 4.52

(s,2H).

EXAMPLE 14

10   **N-4-Cbz-N-1-carboxymethyl cytosine pentafluorophenyl ester (14)**

N-4-Cbz-N-1-carboxymethyl-cytosine   (**13**,   4.00   g;   13.2   mmol)   and

pentafluorophenol (2.67 g; 14.5 mmol) were mixed with DMF (70 ml), cooled to 0°C with

ice-water, and DCC (3.27 g; 15.8 mmol) was added. The ice bath was removed after 3 min

and the mixture was stirred for 3 h at room temperature. The precipitated DCU was

15   removed by filtration, washed with DMF, and the filtrate was evaporated to dryness, *in*

vacuo (0.2 mmHg). The solid residue was treated with methylene chloride (250 ml), stirred

vigorously for 15 min, filtered, washed twice with diluted sodium hydrogen carbonate and

once with saturated sodium chloride, dried over magnesium sulfate, and evaporated to

dryness, *in vacuo*. The solid residue was recrystallized from 2-propanol (150 ml) and the

20   crystals were washed thoroughly with ether. Yield 3.40 g (55%). M.p. 241-245°C. Anal.

for $C_{20}H_{12}N_3F_5O_5$. Found(calc.); C: 51.56(51.18); H: 2.77(2.58); N: 9.24(8.95).^{1}H-NMR

(90   MHz;   CDCl$_3$):   7.66   ppm   (d,J=7.63Hz,1H,H-6);   7.37   (s,5H,Ph);   7.31

(d,J=7.63Hz,1H,H-5); 5.21 (s,2H,PhC$\underline{H}_2$-); 4.97 (s,2H,NC$\underline{H}_2$-). FAB-MS: 470 (M+1)

25   **EXAMPLE 15**

N-(N-Boc-2-aminoethyl)-N-[(N-4-Cbz-1-cytosinyl)acetyl]glycine (15)

To a solution of (N-Boc-2-aminoethyl)glycine **2**, in DMF, prepared as described in

Example **2**, was added triethyl amine (7.00 ml; 50.8 mmol) and N-4-Cbz-N-1-carboxy-

methyl-cytosine pentafluorophenyl ester (**14**, 2.70 g; 5.75 mmol). After stirring the solution

30   for 1 h at room temperature, methylene chloride (150 ml), saturated sodium chloride (250

ml), and 4 N hydrochloric acid to pH ~1 were added. The organic layer was separated and washed twice with saturated sodium chloride, dried over magnesium sulfate, and evaporated to dryness, *in vacuo*, first with a water aspirator and then with an oil pump. The oily residue was treated with water (25 ml) and was again evaporated to dryness, *in vacuo*. This

5 procedure then was repeated. The oily residue (2.80 g) was then dissolved in methylene chloride (100 ml), petroleum ether (250 ml) was added, and the mixture was stirred overnight. The title compound was isolated by filtration and washed with petroleum ether. Tlc (system 1) indicated substantial quantities of pentafluorophenol, but no attempt was made to remove it. Yield: 1.72 g (59%). M.p. 156°C(decomp.). ^{1}H-NMR (250 MHz,

10 CDCl$_3$): Due to the limited rotation around the secondary amide bond several of the signals were doubled in the ratio 2:1,(indicated in the list by mj. for major and mi. for minor). 7.88 ppm (dd,1H,H-6); 7.39 (m,5H,Ph); 7.00 (dd,1H,H-5); 6.92 (b,1H,BocN$\underline{H}$); 6.74 (b,1H,ZN$\underline{H}$)-?; 5.19 (s,2H,Ph-C$\underline{H}_3$); 4.81 ppm (s, mj., Cyt-CH$_2$-CO-); 4.62 ppm (s, mi., Cyt-CH$_2$-CO-); 4.23 (s, mi., CONRC$\underline{H}_2$CO$_2$H); 3.98 ppm (s, mj., CONRC$\underline{H}_2$CO$_2$H); 3.42-3.02

15 (unres. m, -CH$_2$CH$_2$- and water);1.37 (s,9H,tBu). FAB-MS: 504 (M+1); 448 (M+1-tBu).

EXAMPLE 16

N-(N-Boc-2-aminoethyl)-N-[(N-4-Cbz-1-cytosinyl)acetyl]glycine

pentafluorophenyl ester (16)

20 N-(N-Boc-2-aminoethyl)-N-[(N-4-Cbz-1-cytosinyl)acetyl]glycine (**15**, 1.50 g; 2.98 mmol) and pentafluorophenol (548 mg; 2.98 mmol) was dissolved in DMF (10 ml) Methylene chloride (10 ml) was added, the reaction mixture was cooled to 0°C in an ice bath, and DCC (676 mg; 3.28 mmol) was added. The ice bath was removed after 3 min and the mixture was stirred for 3 h at ambient temperature. The precipitate was isolated by

25 filtration and washed once with methylene chloride. The precipitate was dissolved in boiling dioxane (150 ml) and the solution was cooled to 15°C, whereby DCU precipitated. The DCU was removed by filtration and the resulting filtrate was poured into water (250 ml) at 0°C. The title compound was isolated by filtration, was washed with water, and dried over an appropriate drying agent, *in vacuo*. Yield 1.30 g (65%). Analysis for

30 C$_{29}$H$_{28}$N$_5$O$_8$F$_5$. Found(calc.); C: 52.63(52.02); H: 4.41(4.22); N: 10.55(10.46). ^{1}H-NMR

(250 MHz; DMSO-d$_6$): showed essentially the spectrum of the above acid, most probably due to hydrolysis of the ester. FAB-MS: 670 (M+1); 614 (M+1-tBu)

EXAMPLE 17

N-(N-Boc-2-aminoethyl)-N-[(N-4-Cbz-1-cytosinyl)acetyl]glycine

(17)

N-(N-Boc-2-aminoethyl)glycine ethyl ester (**8**, 5.00 g; 20.3 mmol), DhbtOH (3.64 g; 22.3 mmol) and N-4-Cbz-N-1-carboxymethyl cytosine (**13**, 6.77 g; 22.3 mmol) were suspended in DMF (100 ml). Methylene chloride (100 ml) was then added. The solution was cooled to 0°C and DCC (5.03 g; 24.4 mmol) was added. The ice bath was removed after 2 h and stirring was continued for another hour at ambient temperature. The reaction mixture was evaporated to dryness, *in vacuo*. The residue was suspended in ether (100 ml) and stirred vigorously for 30 min. The solid material was isolated by filtration and the ether wash procedure was repeated twice. The material was then stirred vigorously for 15 min with dilute sodium hydrogencarbonate (aprox. 4% solution, 100 ml), filtered and washed with water. This procedure was then repeated once, which after drying left 17.0 g of yellowish solid material. The solid was then boiled with dioxane (200 ml) and filtered while hot. After cooling, water (200 ml) was added. The precipitated material was isolated by filtration, washed with water, and dried. According to HPLC (observing at 260 nm) this material has a purity higher than 99%, besides the DCU. The ester was then suspended in THF (100 ml), cooled to 0°C, and 1 N LiOH (61 ml) was added. After stirring for 15 minutes, the mixture was filtered and the filtrate was washed with methylene chloride (2 x 150 ml). The alkaline solution then was cooled to 0°C and the pH was adjusted to 2.0 with 1 N HCl. The title compound was isolated by filtration and was washed once with water, leaving 11.3 g of a white powder after drying. The material was suspended in methylene chloride (300 ml) and petroleum ether (300 ml) was added. Filtration and wash afforded 7.1 g (69%) after drying. HPLC showed a purity of 99% R$_T$= 19.5 min, and a minor impurity at 12.6 min (approx. 1%) most likely the Z-deprotected monomer. Anal. for C$_{23}$H$_{29}$N$_5$O$_8$ found(calc.) C: 54.16(54.87); H: 5.76(5.81) and N: 13.65(13.91). ^{1}H-NMR (250 MHz, DMSO-d$_6$): 10.78 (b.s, 1H, CO$_2$H); 7.88 (2 overlapping doublets, 1H, Cyt H-5);

7.41-7.32 (m, 5H, Ph); 7.01 (2 overlapping doublets, 1H, Cyt H-6); 6.94 & 6.78 (unres. triplets, 1H, BocN$\underline{H}$); 5.19 (s, 2H, PhC$\underline{H}_2$); 4.81 & 4.62 (s, 2H, C$\underline{H}_2$CON); 4.17 & 3.98 (s, 2H, C$\underline{H}_2$CO$_2$

EXAMPLE 18

9-Carboxymethyl adenine ethyl ester (18)

Adenine (10.0 g, 74 mmol) and potassium carbonate (10.29 g, 74.0 mmol) were suspended in DMF and ethyl bromoacetate (8.24 ml, 74 mmol) was added. The suspension was stirred for 2.5 h under nitrogen at room temperature and then filtered. The solid residue was washed three times with DMF (10 ml). The combined filtrate was evaporated to dryness, *in vacuo*. The yellow-orange solid material was poured into water (200 ml) and 4 N HCl was added to pH≈6. After stirring at 0°C for 10 min, the solid was filtered off, washed with water, and recrystallized from 96% ethanol (150 ml). The title compound was isolated by filtration and washed thoroughly with ether. Yield 3.4 g (20%). M.p. 215.5-220°C. Anal. for C$_9$H$_{11}$N$_5$O$_2$ found(calc.): C: 48.86(48.65); H: 5.01(4.91); N: 31.66(31.42). ^{1}H-NMR (250 MHz; DMSO-d$_6$): (s, 2H, H-2 & H-8), 7.25 (b. s., 2H, NH$_2$), 5.06 (s, 2H, NCH$_2$), 4.17 (q, 2H, J=7.11 Hz, OCH$_2$) and 1.21 (t, 3H, J=7.13 Hz, NCH$_2$). ^{13}C-NMR. 152.70, 141.30, 61.41, 43.97 and 14.07. FAB-MS. 222 (MH+). IR: Frequency in cm^{-1} (intensity). 3855 (54.3), 3274(10.4), 3246(14.0), 3117(5.3), 2989(22.3), 2940(33.9), 2876(43.4), 2753(49.0), 2346(56.1), 2106(57.1), 1899(55.7), 1762(14.2), 1742(14.2), 1742(1.0), 1671(1.8), 1644(10.9), 1606(0.6), 1582(7.1), 1522(43.8), 1477(7.2), 1445(35.8) and 1422(8.6). The position of alkylation was verified by X-ray crystallography on crystals, which were obtained by recrystallization from 96% ethanol.

Alternatively, 9-carboxymethyl adenine ethyl ester **18**, can be prepared by the following procedure. To a suspension of adenine (50.0 g, 0.37 mol) in DMF (1100 ml) in 2 L three-necked flask equipped with a nitrogen inlet, a mechanical stirrer and a dropping funnel was added 16.4 g (0.407 mol) hexane washed sodium hydride- mineral oil dispersion. The mixture was stirred vigorously for 2 hours, then ethyl bromacetate 75 ml, 0.67 mol) was added dropwise over the course of 3 hours. The mixture was stirred for one additional hour, whereafter tlc indicated complete conversion of adenine. The mixture was

evaporated to dryness at 1 mm Hg and water (500 ml) was added to the oily residue which caused crystallization of the title compound. the solid was recrystallized from 60% ethanol (600 ml). Yield after drying 53.7 (65.6%). HPLC (215 nm) purity > 99.5%.

5   **EXAMPLE 19**

N-6-Cbz-9-carboxymethyl adenine ethyl ester (19)

9-Carboxymethyladenine ethyl ester (**18**, 3.40 g, 15.4 mmol) was dissolved in dry DMF (50 ml) by gentle heating, cooled to 20°C, and added to a solution of N-ethyl-Cbz-imidazole tetrafluoroborate (62 mmol) in methylene chloride (50 ml) over a period of 15

10   min with ice-cooling. Some precipitation was observed. The ice bath was removed and the solution was stirred overnight. The reaction mixture was treated with saturated sodium hydrogen carbonate (100 ml). After stirring for 10 min, the phases were separated and the organic phase was washed successively with one volume of water, dilute potassium hydrogen sulfate (twice), and with saturated sodium chloride. The solution was dried over

15   magnesium sulfate and evaporated to dryness, *in vacuo*, which afforded 11 g of an oily material. The material was dissolved in methylene chloride (25 ml), cooled to 0°C, and precipitated with petroleum ether (50 ml). This procedure was repeated once to give 3.45 g (63%) of the title compound. M.p. 132-35°C. Analysis for $C_{17}H_{17}N_5O_4$ found (calc.): C: 56.95(57.46); H: 4.71(4.82); N: 19.35(19.71). ^{1}H-NMR (250 MHz; CDCl$_3$): 8.77 (s, 1H, H-

20   2 or H-8); 7.99 (s, 1H, H-2 or H-8); 7.45-7.26 (m, 5H, Ph); 5.31 (s, 2H, N-C$\underline{H}_2$); 4.96 (s, 2H, Ph-C$\underline{H}_2$); 4.27 (q, 2H, J=7.15 Hz, C$\underline{H}_2$CH$_3$) and 1.30 (t, 3H, J=7.15 Hz, CH$_2$C$\underline{H}_3$). ^{13}C-NMR: 153.09; 143.11; 128.66; 67.84; 62.51; 44.24 and 14.09. FAB-MS: 356 (MH+) and 312 (MH+-CO$_2$). IR: frequency in cm^{-1} (intensity). 3423 (52.1); 3182 (52.8); 3115(52.1); 3031(47.9); 2981(38.6); 1747(1.1); 1617(4.8); 15.87(8.4); 1552(25.2); 1511(45.2);

25   1492(37.9); 1465(14.0) and 1413(37.3).

EXAMPLE 20

N-6-Cbz-9-carboxymethyl adenine (20)

N-6-Cbz-9-carboxymethyladenine ethyl ester (**19**, 3.20 g; 9.01 mmol) was mixed

30   with methanol (50 ml) cooled to 0°C. Sodium Hydroxide Solution (50 ml; 2N) was added,

whereby the material quickly dissolved. After 30 min at 0°C, the alkaline solution was washed with methylene chloride (2x50ml). The aqueous solution was brought to pH 1.0 with 4 N HCl at 0°C, whereby the title compound precipitated. The yield after filtration, washing with water, and drying was 3.08 g (104%). The product contained salt and

5 elemental analysis reflected that. Anal. for $C_{15}H_{13}N_5O_4$ found(calc.): C: 46.32(55.05); H: 4.24(4.00); N: 18.10(21.40) and C/N: 2.57(2.56). ^{1}H-NMR(250 MHz; DMSO-d_6): 8.70 (s, 2H, H-2 and H-8); 7.50-7.35 (m, 5H, Ph); 5.27 (s, 2H, N-C$\underline{H}_2$); and 5.15 (s, 2H, Ph-C$\underline{H}_2$). ^{13}C-NMR. 168.77, 152.54, 151.36, 148.75, 145.13, 128.51, 128.17,127.98, 66.76 and 44.67.IR (KBr) 3484(18.3); 3109(15.9); 3087(15.0); 2966(17.1); 2927(19.9); 2383(53.8);

10 1960(62.7); 1739(2.5); 1688(5.2); 1655(0.9); 1594(11.7); 1560(12.3); 1530(26.3); 1499(30.5); 1475(10.4); 1455(14.0); 1429(24.5) and 1411(23.6). FAB-MS: 328 (MH+) and 284 (MH+-CO_2). HPLC (215 nm, 260 nm) in system 1: 15.18 min, minor impurities all less than 2%.

15 **EXAMPLE 21**

N-(N-Boc-2-aminoethyl)-N-[(N-6-Cbz-9-adeninyl)acetyl]glycine

ethyl ester (21)

N-(N-Boc-2-aminoethyl)glycine ethyl ester (**8**, 2.00 g; 8.12 mmol), DhbtOH (1.46 g; 8.93 mmol) and N-6-Cbz-9-carboxymethyl adenine (**20**, 2.92 g; 8.93 mmol) were dissolved

20 in DMF (15 ml). Methylene chloride (15 ml) then was added. The solution was cooled to 0°C in an ethanol/ice bath. DCC (2.01 g; 9.74 mmol) was added. The ice bath was removed after 2.5 h and stirring was continued for another 1.5 hour at ambient temperature. The precipitated DCU was removed by filtration and washed once with DMF (15 ml), and twice with methylene chloride (2 x 15 ml). To the combined filtrate was added more

25 methylene chloride (100 ml). The solution was washed successively with dilute sodium hydrogen carbonate (2 x 100 ml), dilute potassium hydrogen sulfate (2 x 100 ml), and saturated sodium chloride (1 x 100 ml). The organic phase was evaporated to dryness, *in vacuo*, which afforded 3.28 g (73%) of a yellowish oily substance. HPLC of the raw product showed a purity of only 66% with several impurities, both more and less polar than

30 the main peak. The oil was dissolved in absolute ethanol (50 ml) and activated carbon was

added. After stirring for 5 minutes, the solution was filtered. The filtrate was mixed with water (30 ml) and was left with stirring overnight. The next day, the white precipitate was removed by filtration, washed with water, and dried, affording 1.16 g (26%) of a material with a purity higher than 98% by HPLC. Addition of water to the mother liquor afforded

5   another 0.53 g with a purity of approx. 95%. Anal. for $C_{26}H_{33}N_7O_7 \cdot H_2O$ found(calc.) C: 55.01(54.44; H: 6.85(6.15) and N: 16.47(17.09). ^{1}H-NMR (250 MHz, CDCl$_3$) 8.74 (s, 1H, Ade H-2); 8.18 (b. s, 1H, ZNH); 8.10 & 8.04 (s, 1H, H-8); 7.46-7.34 (m, 5H, Ph); 5.63 (unres. t, 1H, BocNH); 5.30 (s, 2H, PhCH$_2$); 5.16 & 5.00 (s, 2H, C$\underline{H}_2$CON); 4.29 & 4.06 (s, 2H, C$\underline{H}_2$CO$_2$H); 4.20 (q, 2H, OC$\underline{H}_2$CH$_3$); 3.67-3.29 (m, 4H, C$\underline{H}_2$C$\underline{H}_2$); 1.42 (s, 9H, ^{t}Bu) and

10   1.27 (t, 3H, OCH$_2$C$\underline{H}_3$). The spectrum shows traces of ethanol and DCU.

EXAMPLE 22

N-(N-Boc-2-aminoethyl)-N-[(N-6-Cbz-9-adeninyl)acetyl]glycine (22)

N-(N-Boc-2-aminoethyl)-N-[(N-6-Cbz-9-adeninyl)acetyl]glycine ethyl ester (21,

15   1.48 g; 2.66 mmol) was suspended in THF (13 ml) and the mixture was cooled to 0°C. Lithium hydroxide (8 ml; 1 N) was added. After 15 min of stirring, the reaction mixture was filtered, extra water (25 ml) was added, and the solution was washed with methylene chloride (2 x 25 ml). The pH of the aqueous solution was adjusted to pH 2.0 with 1 N HCl. The precipitate was isolated by filtration, washed with water, and dried, affording 0.82 g

20   (58%). The product reprecipitated twice with methylene chloride/ petroleum ether, 0.77 g (55%) after drying. M.p. 119°C (decomp.) Anal. for $C_{24}H_{29}N_7O_7 \cdot H_2O$ found(calc.) C: 53.32(52.84); H: 5.71(5.73); N: 17.68(17.97). FAB-MS. 528.5 (MH+). ^{1}H-NMR (250 MHz, DMSO-d$_6$). 12.75 (very b, 1H, CO$_2$H); 10.65 (b. s, 1H, ZNH); 8.59 (d, 1H, J= 2.14 Hz, Ade H-2); 8.31 (s, 1H, Ade H-8); 7.49-7.31 (m, 5H, Ph); 7.03 & 6.75 (unresol. t, 1H,

25   BocNH); 5.33 & 5.16 (s, 2H, CH$_2$CON); 5.22 (s, 2H, PhC$\underline{H}_2$); 4.34-3.99 (s, 2H, CH$_2$CO$_2$H); 3.54-3.03 (m's, includes water, C$\underline{H}_2$C$\underline{H}_2$) and 1.39 & 1.37 (s, 9H, ^{t}Bu). ^{13}C-NMR. 170.4; 166.6; 152.3; 151.5; 149.5; 145.2; 128.5; 128.0; 127.9; 66.32; 47.63; 47.03; 43.87 and 28.24.

30   **EXAMPLE 23**

2-Amino-6-chloro-9-carboxymethylpurine (23)

To a suspension of 2-amino-6-chloropurine (5.02 g; 29.6 mmol) and potassium carbonate (12.91 g; 93.5 mmol) in DMF (50 ml) was added bromoacetic acid (4.70 g; 22.8 mmol). The mixture was stirred vigorously for 20 h. under nitrogen. Water (150 ml) was added and the solution was filtered through Celite to give a clear yellow solution. The solution was acidified to a pH of 3 with 4 N hydrochloric acid. The precipitate was filtered and dried, *in vacuo*, over an appropriate drying agent. Yield (3.02 g; 44.8%). ^{1}H-NMR(DMSO-d6): d = 4.88 ppm (s,2H); 6.95 (s,2H); 8.10 (s,1H).

EXAMPLE 24

2-Amino-6-benzyloxy-9-carboxymethylpurine (24)

Sodium (2.0 g; 87.0 mmol) was dissolved in benzyl alcohol (20 ml) and heated to 130°C for 2 h. After cooling to 0°C, a solution of 2-amino-6-chloro-9-carboxymethylpurine (**23**, 4.05 g; 18.0 mmol) in DMF (85 ml) was slowly added, and the resulting suspension stirred overnight at 20°C. Sodium hydroxide solution (1N, 100 ml) was added and the clear solution was washed with ethyl acetate (3 x 100 ml). The water phase then was acidified to a pH of 3 with 4 N hydrochloric acid. The precipitate was taken up in ethyl acetate (200 ml), and the water phase was extracted with ethyl acetate (2 x 100 ml). The combined organic phases were washed with saturated sodium chloride solution (2 x 75 ml), dried with anhydrous sodium sulfate, and taken to dryness by evaporation, *in vacuo*. The residue was recrystallized from ethanol (300 ml). Yield after drying, *in vacuo*, over an appropriate drying agent: 2.76 g (52%). M.p. 159-65°C. Anal. (calc., found) C(56.18; 55.97), H(4.38; 4.32), N(23.4; 23.10). ^{1}H-NMR (DMSO-d$_6$): 4.82 ppm.(s,2H); 5.51 (s,2H); 6.45 (s,2H); 7.45 (m,5H); 7.82 (s,1H).

EXAMPLE 25

N-(N-Boc-2-aminoethyl)-N-[(2-Amino-6-benzyloxy-purine-9-yl)acetyl]glycine [BocGaeg monomer] (25)

2-Amino-6-benzyloxy-9-carboxymethyl-purine (**24**, 0.50 g; 1.67 mmol), N-Boc-aminoethyl glycine methyl ester (0.65 g; 2.80 mmol), diisopropylethyl amine (0.54 g; 4.19 mmol), and bromo-tris-pyrrolidino-phosphonium-hexafluoro-phosphate (PyBroP®) (0.798

g; 1.71 mmol) were stirred in DMF (2 ml) for 4 h. The clear solution was poured into an ice-cooled solution of sodium hydrogen carbonate (1 N; 40 ml) and extracted with ethyl acetate (3 X 40 ml). The organic layer was washed with potassium hydrogen sulfate solution (1 N; 2 X 40 ml), sodium hydrogen carbonate (1 N; 1 X 40 ml) and saturated sodium chloride solution (60 ml). After drying with anhydrous sodium sulfate and evaporation, *in vacuo*, the solid residue was recrystallized from ethyl acetate/hexane (20 ml (2:1)) to give the methyl ester in 63% yield (MS-FAB 514 (M+1). Hydrolysis was accomplished by dissolving the ester in ethanol/water (30 ml (1:2)) containing conc. sodium hydroxide (1 ml). After stirring for 2 h, the solution was filtered and acidified to a pH of 3, by the addition of 4 N hydrochloric acid. The title compound was obtained by filtration. Yield: 370 mg (72% for the hydrolysis). Purity by HPLC was more than 99%. Due to the limited rotation around the secondary amide several of the signals were doubled in the ratio 2:1 (indicated in the list by mj. for major and mi. for minor). ^{1}H-NMR(250, MHz, DMSO-d_6): d = 1.4 ppm. (s,9H); 3.2 (m,2H); 3.6 (m,2H); 4.1 (s, mj., CONRC$\underline{H}_2$COOH); 4.4 (s, mi., CONRC$\underline{H}_2$COOH); 5.0 (s, mi., Gua-C$\underline{H}_2$CO-); 5.2 (s, mj., Gua-C$\underline{H}_2$CO); 5.6 (s,2H); 6.5 (s,2H); 6.9 (m, mi., BocNH); 7.1 (m, mj., BocNH); 7.5 (m.,3H); 7.8 (s,1H); 12,8 (s;1H). ^{13}C-NMR. 170.95; 170.52; 167.29; 166.85; 160.03; 159.78; 155.84; 154.87; 140.63; 136.76; 128.49; 128.10; 113.04; 78.19; 77.86; 66.95; 49.22; 47.70; 46.94; 45.96; 43.62; 43.31 and 28.25.

EXAMPLE 26

Synthesis of PNA Oligomers by Solid Phase, General Procedure

The functionalized resin is measured out to typically provide 0.1-1.0 millimoles of functionality, (functionalities attached to resins are commercially available through various sources e.g. Peptides International, Kentucky). This weight of resin is suspended in a 1:1 (v:v) dichloromethane:dimethylformamide solution (30mL/1g of resin) and allowed to swell for a period of time if desired. The solvent is then removed by filtration and the resin resuspended in trifluoroacetic acid (1mL/1gm of resin) and shaken for 3 minutes. The trifluoroacetic acid is removed by filtration and this step is repeated twice. The resin is washed three times with a solution of 1:1 (v:v) dichloromethane:dimethylformamide. The

resulting resin is resuspended in pyridine solution (5mL/100mg of resin) and vacuum

filtered to remove the pyridine. This step is repeated twice. The resin is suspended in 1:1

(v:v) pyridine:dimethylformamide and to this suspension is added the desired PNA

monomer (2-10 molar equivalents), TBTU (1.9-9.9 molar equivalents), and di-

5   isopropylethylamine (5-20 molar equivalents) such that the final concentration of PNA

monomer is 0.2M. The suspension is shaken for 15-60 minutes and the spent coupling

solution is removed by filtration. The resin is washed with pyridine three times, and any

unreacted amines are capped using Rapoport's Reagent, 5 equivalents in DMF for 5

minutes. The resin is then washed three times with pyridine followed by three washes with

10   a solution of 1:1 (v:v) dichloromethane: dimethylformamide (5mL/100mg of resin). At this

point, the resin is ready for the next coupling reaction and this procedure is repeated until

the desired PNA is assembled on the resin.

Specific Examples of Amino Ethyl Glycine (aeg-) PNAs and aeg-PNA Derivatives

15   **Prepared by this General Method**

Resin Employed	aeg-PNA/aeg-PNA Derivative Prepared
Merrifield	H_2N-GCAT-COOH (SEQ ID NO:1)
Lys Substituted Merrifield	H_2N-GCAT-Lys-COOH (SEQ ID NO:2)
MBHA	H_2N-GCAT-CONH$_2$ (SEQ ID NO:3)
Lys Substituted MBHA	H_2N-GCAT-Lys-CONH$_2$ (SEQ ID NO:4)

EXAMPLE 27

30   **Capping of the PNA**

PNA can be capped by a non-PNA moiety on the N terminus by following the procedures described in Example **26** and substituting a desired carboxylic acid-based capping reagent for the PNA monomer in the final coupling step.

5   **Specific Examples of aeg-PNAs and aeg-PNA Derivatives Prepared by this General Method**

Resin Employed	aeg-PNA/aeg-PNA Derivative Prepared
10

| **Capping Reagent = Acetyl** | |
| Merrifield | CH_3CONH-GCAT-COOH (SEQ ID NO:5) |

15

| Lys Substituted Merrifield | CH_3CONH-GCAT-Lys-COOH (SEQ ID NO:6) |

| MBHA | CH_3CONH-GCAT-$CONH_2$ (SEQ ID NO:7) |
20

| Lys Substituted MBHA | CH_3CONH-GCAT-Lys-$CONH_2$ (SEQ ID NO:8) |

25

| **Capping Reagent = N-Boc glycine** | |
| Merrifield | BocGly-GCAT-COOH (SEQ ID NO:9) |

30

Lys Substituted   BocGly-GCAT-Lys-COOH (SEQ ID NO:10)
Merrifield

MBHA   BocGly-GCAT-CONH$_2$ (SEQ ID NO:11)

5

Lys Substituted   BocGly-GCAT-Lys-CONH$_2$ (SEQ ID NO:12)
MBHA

10   **Capping Reagent = 1. Glycine; 2. Cholic Acid (ChA)**

Merrifield   ChA-GlyGCAT-COOH (SEQ ID NO:13)

Lys Substituted   ChA-GlyGCAT-Lys-COOH (SEQ ID NO:14)
15   Merrifield

MBHA   ChA-GlyGCAT-CONH$_2$ (SEQ ID NO:15)

20   Lys Substituted   ChA-GlyGCAT-Lys-CONH$_2$ (SEQ ID NO:16)
MBHA

Capping Reagent = Fluorescein (Fluor)

25   Merrifield   Fluor-GCAT-COOH (SEQ ID NO:17)

Lys Substituted   Fluor-GCAT-Lys-COOH (SEQ ID NO:18)
Merrifield

30   MBHA   Fluor-GCAT-CONH$_2$ (SEQ ID NO:19)

Lys Substituted   Fluor-GCAT-Lys-CONH$_2$ (SEQ ID NO:20)
MBHA

5

Capping Reagent = 1.Glycine, 2.Fluorescein (Fluor)

Merrifield   Fluor-GlyGCAT-COOH (SEQ ID NO:21)

10   Lys Substituted   Fluor-GlyGCAT-Lys-COOH (SEQ ID
      Merrifield   NO:22)

MBHA   Fluor-GlyGCAT-CONH$_2$ (SEQ ID NO:23)

15

Lys Substituted   Fluor-GlyGCAT-Lys-CONH$_2$ (SEQ ID
MBHA   NO:24)

20

**Capping Reagent = Adipic Acid t-butyl ester
(tBuOOC(CH$_2$)$_4$CO)**

Merrifield   tBuOOC(CH$_2$)$_4$CONH-GCAT-COOH (SEQ ID
25   NO:25)

Lys Substituted   tBuOOC(CH$_2$)$_4$CONH-GCAT-Lys-COOH (SEQ
Merrifield   ID NO:26)

30

| MBHA | tBuOOC(CH$_2$)$_4$CONH-GCAT-CONH$_2$ (SEQ ID NO:27) |

5

| Lys Substituted MBHA | tBuOOC(CH$_2$)$_4$CONH-GCAT-Lys-CONH$_2$ (SEQ ID NO:28) |

Capping Reagent = Cholic Acid (ChA)

10

| Merrifield | ChA-GCAT-COOH (SEQ ID NO:29) |

| Lys Substituted Merrifield | ChA-GCAT-Lys-COOH (SEQ ID NO:30) |

15

| MBHA | ChA-GCAT-CONH$_2$ (SEQ ID NO:31) |

| Lys Substituted MBHA | ChA-GCAT-Lys-CONH$_2$ (SEQ ID NO:32) |

20   **EXAMPLE 28**

General Cleavage Reaction

The completed PNA or PNA derivative may be cleaved from the resin and isolated via the following. The resin is washed twice with trifluoroacetic acid, then suspended in a mixture of thioanisole, m-cresol, trifluoromethanesulfonic acid, and trifluoroacetic acid in a

25   ratio of 1:1:2:6, 200 µl/1 µmole, for 1 hour at room temperature. The cleavage solution is then drained into ether, 1 ml/µmole, and the above process is repeated. The precipitate is then collected by either filtration or centrifugation.

EXAMPLE 29

30   **General Purification Procedure**

The isolated PNA or PNA derivative is purified by preparative reverse phase HPLC using a Waters μBondapak

Phenyl-C18 column (19mmX150mm) and eluting with a gradient comprised of a solution of 9:1 acetonitrile:0.050M NH_4OAc, pH 6, .1% hexafluoroisopropanol added to a solution

5   of 0.050M NH_4OAc, pH 6, .1% hexafluoroisopropanol. The eluent can be adjusted for the particular PNA or PNA derivative being purified.

EXAMPLE 30

N-[(N-phthaloyl)(1-methyl-O-benzyl)-2-aminoethyl]-N-[(1-thyminyl)acetyl]glycine

10   **(30)**

A. (O-Benzyl) Boc Serine methyl ester (30A)

(O-benzyl) Boc Serine 2.00 g (6.8 mmoles) was dissolved in 50 ml of ether. To this solution was added an ether solution containing freshly prepared diazomethane prepared from 5 g of Diazald using the procedure from Aldrich Technical Bulletin number AL-180.

15   After stirring the solution for 30 minutes, glacial acetic acid was added dropwise until the solution became clear. The resulting solution was concentrated in vacuo to yield 2.00 g, 95 % as a clear oil. ^{1}H: (CDCl$_3$); 7.3 (d, 5H), 5.5 (bs, 1H), 4.5 (s, 2H), 3.8 (m, 2 H), 3.7 (s, 3 H), 1.4 (s, 9H)

B.   1-O-Benzyl-2-N-Boc-2,3-propandiol (30B)

20   Compound **30A** was dissolved in 50 ml of methanol and cooled to 0 °C. To this was added 300 mg of lithium borohydride in small portions, waiting for the gas evolution to cease between additions. When all of the hydride had been added the reaction was refluxed for 1 hour. It was then cooled to 0°C, and quenched by the dropwise addition of saturated sodium bicarbinate. The solution was extracted twice with 50 ml portions of chloroform.

25   The combined organic extracts were washed again with saturated sodium bicarbonate, followed by saturated sodium chloride solutions. The chloroform solution was dried over magnesium sulfate and concentrated to an oil. The oil was chromatographed over silica gel eluting with 7% methanol in dichloromethane, isolating 1.30 g as a clear oil. 1H: (CDCl3); 7.3 (s, 5 H), 5.2 (bs, 1 H), 4.5 (s, 2 H), 3.9-3.5 (mm, 5 H), 1.4 (s, 9 H)

C.   1-O-benzyl-2-N-Boc-3-phthaloyl-2,3-diaminopropanol (30C)

Compound **30B** was dissolved in 100 ml of freshly distilled THF. To this solution was added the following reagents; triphenyl phosphine, (1.26 g 4.82 mmol), diethyl azodicarboxylate (0.839 g 4.82 mmol), and phthalimide (0.710 g 4.82 mmol), and the reaction stirred overnight at room temperature. After stirring the solution was concentrated to a yellow solid and chromatographed over silica gel using 30% ethyl acetate in hexanes. The product was isolated as a white solid weighing 1.60 g, 97 %. 1H: (CDCl3); 7.9 (m, 2 H), 7.7 (m, 2H), 7.3 (s, 5 H), 5.1 (d, 1 H), 4.5 (s, 2 H), 4.0-3.5 (mm, 5 H), 1.3 (s, 9 H).

D.   N-[(N-phthaloyl)(1-methyl-O-benzyl)-2-aminoethyl]glycine methyl ester (30D)

Compound **30C** (1.00 g, 2.9 mmol) is dissolved in 20 ml dichloromethane and cooled to 0°C. To this is added 20ml TFA and the reaction is allowed to warm to room temperature. Upon completion, the reaction is concentrated in vacuo and then redissolved in dichloromethane and washed with 1 N NaOH and brine, the organic solution is dried and concentrated to give the free amine. This amine is then dissolved in anhydrous THF with 2 eq of triethylamine and cooled to 0°C. Methyl bromoacetate (0.500 g, 3.2 mmol) is added and the reaction is allowed to stir at room temperature until complete. Standard aqueous workup and chromatography yields the title compound.

E.   N-[(N-phthaloyl)(1-methyl-O-benzyl)-2-aminoethyl]-N-[(1-thymin-yl)acetyl]glycine (30)

The functionalized backbone monomer of Example **30D** is dissolved in THF and treated with 3 eq of triethyl amine. To this 1 eq of 1-chlorocarbonylmethyl thymine is added dropwise as a THF solution over 30 min. The resulting solution is stirred overnight or until complete. Standard acidic workup and chromatography yield the title compound.

EXAMPLE 31

N-[(N-phthaloyl)(1-methyl-O-benzyl)-2-aminoethyl]-N-[(9-adeninyl)acetyl]glycine (31)

A.   9-Carboxymethyl adenine ethyl ester (31A)

Adenine (10.0 g, 74 mmol) and potassium carbonate (10.29 g, 74.0 mmol) were suspended in DMF and ethyl bromoacetate (8.24 ml, 74 mmol) was added. The suspension

was stirred for 2.5 h under nitrogen at room temperature and then filtered. The solid residue was washed three times with DMF (10 ml). The combined filtrate was evaporated to dryness, *in vacuo*. The yellow-orange solid material was poured into water (200 ml) and 4 N HCl was added to pH≈6. After stirring at 0°C for 10 min, the solid was filtered off,

5   washed with water, and recrystallized from 96% ethanol (150 ml). The title compound was isolated by filtration and washed thoroughly with ether. Yield 3.4 g (20%). M.p. 215.5-220°C. Anal. for $C_9H_{11}N_5O_2$ found(calc.): C: 48.86(48.65); H: 5.01(4.91); N: 31.66(31.42). ^{1}H-NMR (250 MHz; DMSO-d_6): (s, 2H, H-2 & H-8), 7.25 (b. s., 2H, NH_2), 5.06 (s, 2H, NCH_2), 4.17 (q, 2H, J=7.11 Hz, OCH_2) and 1.21 (t, 3H, J=7.13 Hz, NCH_2). ^{13}C-NMR.

10   152.70, 141.30, 61.41, 43.97 and 14.07. FAB-MS. 222 (MH+). IR: Frequency in cm^{-1} (intensity). 3855 (54.3), 3274(10.4), 3246(14.0), 3117(5.3), 2989(22.3), 2940(33.9), 2876(43.4), 2753(49.0), 2346(56.1), 2106(57.1), 1899(55.7), 1762(14.2), 1742(14.2), 1742(1.0), 1671(1.8), 1644(10.9), 1606(0.6), 1582(7.1), 1522(43.8), 1477(7.2), 1445(35.8) and 1422(8.6). The position of alkylation was verified by X-ray crystallography on crystals,

15   which were obtained by recrystallization from 96% ethanol.

Alternatively, 9-carboxymethyl adenine ethyl ester can be prepared by the following procedure. To a suspension of adenine (50.0 g, 0.37 mol) in DMF (1100 ml) in 2 L three-necked flask equipped with a nitrogen inlet, a mechanical stirrer and a dropping funnel was added 16.4 g (0.407 mol) hexane washed sodium hydride- mineral oil dispersion. The

20   mixture was stirred vigorously for 2 hours, whereafter ethyl bromacetate 75 ml, 0.67 mol) was added dropwise over the course of 3 hours. The mixture was stirred for one additional hour, whereafter tlc indicated complete conversion of adenine. The mixture was evaporated to dryness at 1 mmHg and water (500 ml) was added to the oily residue which caused crystallization of the title compound. The solid was recrystallized from 96% ethanol (600

25   ml). Yield after drying 53.7 (65.6%). HPLC (215 nm) purity > 99.5%.

B.   N-6-Cbz-9-carboxymethyl adenine ethyl ester (31B)

9-Carboxymethyladenine ethyl ester (**31A**, 3.40 g, 15.4 mmol) was dissolved in dry DMF (50 ml) by gentle heating, cooled to 20°C, and added to a solution of N-ethyl-Cbz-imidazole tetrafluoroborate (62 mmol) in methylene chloride (50 ml) over a period of 15

30   min with ice-cooling. Some precipitation was observed. The ice bath was removed and the

solution was stirred overnight. The reaction mixture was treated with saturated sodium hydrogen carbonate (100 ml). After stirring for 10 min, the phases were separated and the organic phase was washed successively with one volume of water, dilute potassium hydrogen sulfate (twice), and with saturated sodium chloride. The solution was dried over magnesium sulfate and evaporated to dryness, *in vacuo*, which afforded 11 g of an oily material. The material was dissolved in methylene chloride (25 ml), cooled to 0°C, and precipitated with petroleum ether (50 ml). This procedure was repeated once to give 3.45 g (63%) of the title compound. M.p. 132-35°C. Analysis for $C_{17}H_{17}N_5O_4$ found (calc.): C: 56.95(57.46); H: 4.71(4.82); N: 19.35(19.71). ^{1}H-NMR (250 MHz; CDCl$_3$): 8.77 (s, 1H, H-2 or H-8); 7.99 (s, 1H, H-2 or H-8); 7.45-7.26 (m, 5H, Ph); 5.31 (s, 2H, N-C$\underline{H}_2$); 4.96 (s, 2H, Ph-C$\underline{H}_2$); 4.27 (q, 2H, J=7.15 Hz, C$\underline{H}_2$CH$_3$) and 1.30 (t, 3H, J=7.15 Hz, CH$_2$C$\underline{H}_3$). ^{13}C-NMR: 153.09; 143.11; 128.66; 67.84; 62.51; 44.24 and 14.09. FAB-MS: 356 (MH+) and 312 (MH+-CO$_2$). IR: frequency in cm^{-1} (intensity). 3423 (52.1); 3182 (52.8); 3115(52.1); 3031(47.9); 2981(38.6); 1747(1.1); 1617(4.8); 15.87(8.4); 1552(25.2); 1511(45.2); 1492(37.9); 1465(14.0) and 1413(37.3).

C.   N-6-Cbz-9-carboxymethyl adenine (31C)

N-6-Cbz-9-carboxymethyladenine ethyl ester (**31B**, 3.20 g; 9.01 mmol) was mixed with methanol (50 ml) cooled to 0°C. Sodium hydroxide solution (50 ml; 2N) was added, whereby the material quickly dissolved. After 30 min at 0°C, the alkaline solution was washed with methylene chloride (2x50ml). The aqueous solution was brought to pH 1.0 with 4 N HCl at 0°C, whereby the title compound precipitated. The yield after filtration, washing with water, and drying was 3.08 g (104%). The product contained salt and elemental analysis reflected that. Anal. for $C_{15}H_{13}N_5O_4$ found(calc.): C: 46.32(55.05); H: 4.24(4.00); N: 18.10(21.40) and C/N: 2.57(2.56). ^{1}H-NMR(250 MHz; DMSO-d$_6$): 8.70 (s, 2H, H-2 and H-8); 7.50-7.35 (m, 5H, Ph); 5.27 (s, 2H, N-C$\underline{H}_2$); and 5.15 (s, 2H, Ph-C$\underline{H}_2$). ^{13}C-NMR. 168.77, 152.54, 151.36, 148.75, 145.13, 128.51, 128.17,127.98, 66.76 and 44.67.IR (KBr) 3484(18.3); 3109(15.9); 3087(15.0); 2966(17.1); 2927(19.9); 2383(53.8); 1960(62.7); 1739(2.5); 1688(5.2); 1655(0.9); 1594(11.7); 1560(12.3); 1530(26.3); 1499(30.5); 1475(10.4); 1455(14.0); 1429(24.5) and 1411(23.6). FAB-MS: 328 (MH+) and

less than 2%.

D.   N-[(N-phthaloyl)(1-methyl-O-benzyl)-2-aminoethyl]-N-[(9-adenin-yl)acetyl]glycine (31)

N-6-Cbz-9-carboxymethyl adenine (31C) is converted to the acid chloride using

5   standard techniques, and treated according to the procedure of Example 30D to yield the title compound.

EXAMPLE 32

N-[(N-phthaloyl)(1-methyl-O-benzyl)-2-aminoethyl]-N-[(1-cytosinyl)acetyl]glycine (32)

10   **A.   N-4-Cbz cytosine (32A)**

Over a period of about 1 h, Cbz chloride (52 ml; 0.36 mol) was added dropwise to a suspension of cytosine (8, 20.0 g;0.18 mol) in dry pyridine (1000 ml) at 0°C under nitrogen in oven-dried equipment. The solution then was stirred overnight, after which the pyridine suspension was evaporated to dryness, *in vacuo*. Water (200 ml) and 4 N hydrochloric acid

15   were added to reach pH 1. The resulting white precipitate was filtered off, washed with water and partially dried by air suction. The still-wet precipitate was boiled with absolute ethanol (500 ml) for 10 min, cooled to 0°C, filtered, washed thoroughly with ether, and dried, *in vacuo*. Yield 24.7 g (54%). M.p.>250°C. Anal. for $C_{12}H_{11}N_3O_3$. Found(calc.); C: 58.59(58.77); H: 4.55(4.52); N: 17.17(17.13). No NMR spectra were recorded since it was

20   not possible to get the product dissolved.

B.   N-4-Cbz-N-1-carboxymethyl cytosine (32B)

In a three necked round bottomed flask equipped with mechanical stirring and nitrogen coverage was placed methyl bromacetate (7.82 ml;82.6 mmol) and a suspension of N-4-Cbz-cytosine (32A, 21.0 g;82.6 mmol) and potassium carbonate (11.4 g;82.6 mmol) in

25   dry DMF (900 ml). The mixture was stirred vigorously overnight, filtered, and evaporated to dryness, *in vacuo*. Water (300 ml) and 4 N hydrochloric acid (10 ml) were added, the mixture was stirred for 15 minutes at 0°C, filtered, and washed with water (2 x 75 ml). The isolated precipitate was treated with water (120 ml), 2N sodium hydroxide (60 ml), stirred for 30 min, filtered, cooled to 0°C, and 4 N hydrochloric acid (35 ml) was added. The title

methanol (1000 ml) and washed thoroughly with ether. This afforded 7.70 g (31%) of pure

compound. The mother liquor from the recrystallization was reduced to a volume of 200 ml

and cooled to 0°C. This afforded an additional 2.30 g of a material that was pure by tlc but

had a reddish color. M.p. 266-274°C. Anal. for $C_{14}H_{13}N_3O_5$. Found(calc.); C:

5   55.41(55.45); H: 4.23(4.32); N: 14.04(13.86). ^{1}H-NMR (90 MHz; DMSO-d$_6$): 8.02 ppm

(d,J=7.32Hz,1H,H-6); 7.39 (s,5H,Ph); 7.01 (d,J=7.32Hz,1H,H-5); 5.19 (s,2H,PhC$\underline{H}_2$-); 4.52

(s,2H).

C.   N-[(N-phthaloyl)(1-methyl-O-benzyl)-2-aminoethyl]-N-[(1-cytosinyl-

)acetyl]glycine (32)

10   N-4-Cbz-N-1-carboxymethyl cytosine is converted to the acid chloride according to

standard techniques, and treated according to the procedure of Example 30D to yield the

title compound.

EXAMPLE 33

15 N-[(N-phthaloyl)(1-methyl-O-benzyl)-2-aminoethyl]-N-[(2-Amino-6-benzyloxy-purine-

9-yl)acetyl]glycine (33)

A.   2-Amino-6-chloro-9-carboxymethylpurine (33A)

To a suspension of 2-amino-6-chloropurine (5.02 g; 29.6 mmol) and potassium

carbonate (12.91 g; 93.5 mmol) in DMF (50 ml) was added bromoacetic acid (4.70 g; 22.8

20 mmol). The mixture was stirred vigorously for 20 h under nitrogen. Water (150 ml) was

added and the solution was filtered through Celite to give a clear yellow solution. The

solution was acidified to a pH of 3 with 4 N hydrochloric acid. The precipitate was filtered

and dried, *in vacuo*, over an appropriate drying agent. Yield (3.02 g; 44.8%). ^{1}H-

NMR(DMSO-d6): d = 4.88 ppm (s,2H); 6.95 (s,2H); 8.10 (s,1H).

25   B.   2-Amino-6-benzyloxy-9-carboxymethylpurine (33B)

Sodium (2.0 g; 87.0 mmol) was dissolved in benzyl alcohol (20 ml) and heated to

130°C for 2 h. After cooling to 0°C, a solution of 2-amino-6-chloro-9-

carboxymethylpurine (33A, 4.05 g; 18.0 mmol) in DMF (85 ml) was slowly added, and the

resulting suspension stirred overnight at 20°C. Sodium hydroxide solution (1N, 100 ml)

30   was added and the clear solution was washed with ethyl acetate (3 x 100 ml). The water

phase then was acidified to a pH of 3 with 4 N hydrochloric acid. The precipitate was taken up in ethyl acetate (200 ml), and the water phase was extracted with ethyl acetate (2 x 100 ml). The combined organic phases were washed with saturated sodium chloride solution (2 x 75 ml), dried with anhydrous sodium sulfate, and taken to dryness by evaporation, *in vacuo*. The residue was recrystallized from ethanol (300 ml). Yield after drying, *in vacuo*, over an appropriate drying agent: 2.76 g (52%). M.p. 159-65°C. Anal. (calc., found) C(56.18; 55.97), H(4.38; 4.32), N(23.4; 23.10). ^{1}H-NMR (DMSO-d$_6$): 4.82 ppm.(s,2H); 5.51 (s,2H); 6.45 (s,2H); 7.45 (m,5H); 7.82 (s,1H).

**C.	N-[(N-phthaloyl)(1-methyl-O-benzyl)-2-aminoethyl]-N-[(2-Amino-6-benzyloxy-purine-9-yl)acetyl]glycine (33)**

2-Amino-6-benzyloxy-9-carboxymethyl-purine (**33B**, 1.67 mmol), Compound **31D** (2.80 mmol), diisopropylethyl amine (4.19 mmol), and bromo-tris-pyrrolidino-phosphonium-hexafluoro-phosphate (PyBroP®) (1.71 mmol) are stirred in DMF (2 ml) for 4 h. The clear solution is poured into an ice-cooled solution of sodium hydrogen carbonate (1 N; 40 ml) and extracted with ethyl acetate (3 X 40 ml). The organic layer is washed with potassium hydrogen sulfate solution (1 N; 2 X 40 ml), sodium hydrogen carbonate (1 N; 1 X 40 ml) and saturated sodium chloride solution (60 ml). The organic layer is separated, dried with anhydrous sodium sulfate and concentrated *in vacuo*. The solid residue is recrys-tallized from ethyl acetate/hexane (20 ml (2:1)) to give the methyl ester. Hydrolysis is accomplished by dissolving the ester in ethanol/water (30 ml (1:2)) containing conc. sodium hydroxide (1 ml). After stirring for 2 h, the solution is filtered and acidified to a pH of 3, by the addition of 4 N hydrochloric acid. The title compound is obtained by filtration. Purity is judged by HPLC.

EXAMPLE 34

N-[(N-Boc)(2-methyl-O-benzyl)-2-aminoethyl]-N-[(1-thyminyl)acetyl]glycine (34)

**A.	(O-benzyl)2-N-Boc-diaminopropanol (34A)**

Compound **30C** is dissolved in 200 ml ethanol in a 500 ml flask. Hydrazine is added to the stirring reaction mixture. The mixture is heated to 60-65° in an oil bath and refluxed 14 hours. Solvent is evaporated *in vacuo*. The residue is dissolved in dichloromethane (250 ml) and extracted twice with an equal volume of NH$_4$OH. The

organic layer is evaporated to yield the crude compound **34A**. The product may be used without further purification or can be further purified by HPLC. NMR is used to assay product purity.

    B.   **N-[(N-Boc)(2-methyl-O-benzyl)-2-aminoethyl]-N-glycine methyl ester (34B)**

    Compound **34A** is treated with methyl bromoacetate according to the procedure of Example **30B** to yield the title compound.

    C.   **N-[(N-Boc)(2-methyl-O-benzyl)-2-aminoethyl]-N-[(1-thyminyl)-acetyl]glycine (34)**

    Compound **34B** is treated with chlorocarbonylmethylthymine according to the procedure of Example **30C** to yield the title compound.

EXAMPLE 35

N-[(N-Boc)(1-hydroxymethyl)-2-aminoethyl]-N-[(1-thyminyl)-acetyl]glycine (35)

    Compound **30** is treated with hydrazine according to the procedure of Example **34A** to convert the phthalimido group to the free amine, which is then treated with Boc$_2$O/NaOH to yield the N-Boc intermediate which is then treated with H$_2$/Pd/C to yield the title compound.

EXAMPLE 36

N-[(N-Boc)(2-hydroxymethyl)-2-aminoethyl]-N-[(1-thyminyl)-acetyl]glycine (36)

    Compound **34** is treated according to the procedures of Example **35** to yield the title compound.

EXAMPLE 37

N-[(N-Boc)(1-formyl)-2-aminoethyl]-N-[(1-thyminylacetyl]glycine (37A) and N-[(N-Boc)(2-formyl)-2-aminoethyl-N-[(1-thyminyl)acetyl]glycine (37B)

    Compound **35** or **36** is treated with oxalyl chloride/DMSO according to the procedure of Omura and Swern, Tetrahedron **34** 1651 (1978) to yield the respective title compound.

EXAMPLE 38

N-[(N-Boc)(1-aminomethyl)-2-aminoethyl]-N-[(1-thyminyl-acetyl]glycine (38A) and N-[(N-Boc)(2-aminomethyl)-2-aminoethyl]-N-[(1-thyminyl)acetyl]glycine (38B)

Compound **37A** or **37B** is dissolved in 4 ml methanol. Sodium acetate pH 4.0 (2

5   ml), sodium cyanoborohydride (0.02 grams, 0.3 mmol) and ammonia in water (300 μl) are added to the reaction mixture, which is stirred 2 hours, after which it is concentrated *in vacuo*. The residue is dissolved in dichloromethane (50 mL) and washed with an equal volume saturated NaHCO₃. The aqueous layer is washed with dichloromethane and the combined organic extracts washed with an equal volume saturated NaCl. The aqueous layer

10   is washed with dichloromethane and the combined organic layers dried over MgSO₄ and concentrated. The residue is chromatographed on a silica gel column, eluting with a gradient of 50% ethyl acetate in hexanes to 100% ethyl acetate. The desired product (0.15 grams, 80%) elutes with 10% Methanol/90% ethyl acetate.

15   **EXAMPLE 39**

Synthesis of N-(N-Boc-2-aminoethyl)-N-[(1-thyminyl)acetyl]-L-serine(OBn) (39)

   A.   **N-Boc-aminoacetaldehyde (39A)**

3-(Boc-amino)-1,2-propanediol (21g, 110 mmol) was dissolved in water (200ml) and stirred with potassium m-periodate (25.3g, 110 mmol) at room temperature under argon

20   for 2h. The reaction mixture was filtered and washed with methylene chloride (100ml). The aqueous layer was extracted with methylene chloride (3x150ml). The organic extract was washed with brine (50ml), dried and evaporated to dryness to give a light pink oil. This oil was purified by flash column chromatography using silica gel and CH_2Cl_2/ethyl acetate as the eluent. Yield was 14g (80%). ^{1}HNMR (CDCl₃) d 1.45 (s, 9H, t-Butyl), 4.08 (d, 2H,

25   CH_2), 5.24 (bs, 1H, NH) and 9.67 (s, 1H, CHO).

   B.   **N-(N-Boc-2-aminoethyl)-L-serine(O-benzyl) methyl ester (39B)**

Method A: L-Serine(OBn) methyl ester (16.93g, 81 mmol) and N-Boc-aminoacetaldehyde (**39A**) (12.88g, 81 mmol) were dissolved in dry CH_2Cl_2 (200ml) and allowed to stir at room temperature under an atmosphere of argon. Glacial acetic acid (1ml)

30   was added and the mixture was stirred for an additional 15 minutes. Sodium

triacetoxyborohydride (18.99g, 90 mmol) was added at room temperature in one lot. After stirring for 3h under argon, the reaction was diluted with CH_2Cl_2 (100ml). The organic layer was washed first with sat. $NaHCO_3$ (100ml) to pH 7 followed by washing with water (50ml) and then brine (50ml). The organic extract was dried and evaporated to dryness.

5   The residue was purified by flash chromatography using CH_2Cl_2/acetone as the eluent. The fractions having the required product were collected and evaporated to dryness to give 8.0g (28%) of pure product as foam. ^{1}HNMR (CDCl$_3$) δ 1.38 (s, 9H, t-Butyl), 1.95 (bs, 1H, NH), 2.60 (m, 1H, CH_2), 2.80 (m, 1H, CH_2), 3.18 (m, 2H, CH_2), 3.42 (t, 1H), 3.65 (m, 2H, CH_2), 3.72 (s, 3H, OCH_3), 4.50 (s, 2H, OCH_2), 5.04 (bs, 1H, NH) and 7.30 (m, 5H, ArH).

10 Anal. Calcd for $C_{11}H_{22}N_2O_5$: C, 50.37; H, 8.45; N, 10.68. Found: C, 50.60; H, 8.43; N, 10.83.

Method B: L-Serine(OBn) methyl ester (2.6g, 12.44 mmol) and N-Boc-aminoacetaldehyde (39A) (2.07g, 13 mmol) were dissolved in dry CH_3OH (35ml) and placed in a parr bottle. To this solution anhydrous NaOAc (1.77g, 13 mmol) and Pd/C

15 (10%, 0.6g) were added under an atmosphere of argon. The reaction mixture was hydrogenated at 3psi of hydrogen for 2h. The catalyst was filtered, washed with methanol (20ml) and the combined filtrate was evaporated to dryness. The residue was partitioned between CH_2Cl_2 (150ml) and sat. $NaHCO_3$(100ml) and extracted with CH_2Cl_2. The organic extract was washed with water (100ml) and brine, dried and evaporated to dryness. The

20 residue was purified by flash column chromatography using CH_2Cl_2/acetone as the eluent. The pure fractions were collected and evaporated to dryness to give 3.9g(89%) of the product as foam.

C.   N-(N-Boc-2-aminoethyl)-N-[(1-thyminyl)acetyl]-L-serine(OBn) methyl ester (39C)

25   The substrate **39B** (7.0g, 20 mmol) and N-1-carboxymethylthymine (3, 3.68g, 20 mmol) were dissolved in dry DMF/CH_2Cl_2 (1:1; 200ml) and cooled to 0°C in an ice bath under an atmosphere of argon. To this cold solution was added DhbtOH (3.62g, 20 mmol) followed by EDC (4.20g, 22 mmol). The reaction mixture was stirred at room temperature under argon overnight and evaporated to dryness. The residue was dissolved in a mixture of

30   CH_2Cl_2 (200 ml) and water (100ml) and extracted in CH_2Cl_2. The organic extract was

washed with 5% NaHCO$_3$ (100ml), water (100ml) and brine(100ml). The CH$_2$Cl$_2$ layer was dried over anhydrous Na$_2$SO$_4$ and evaporated to dryness. The residue was purified by flash column chromatography over silica gel using CH$_2$Cl$_2$/MeOH as the eluent. The pure fractions were collected and evaporated to give 9g (87%) of the product as a foam. ^{1}HNMR

5   (CDCl$_3$) d 1.42 (s, 9H, t-Butyl), 1.90 (s, 3H, CH_3), 3.32 (m, 2H, CH_2), 3.55 (m, 2H, CH_2), 3.72 (s, 3H, OCH_3), 4.00 (m, 2H, CH_2), 4.32 (m, 1H, CH), 4.50 (s, 2H, OCH_2), 4.52 (m, 2H, CH_2), 5.46 (t, 1H, NH), 6.85 (s, 1H, C$_6H$), 7.30 (m, 5H, ArH) and 8.8 (s, 1H, NH). Anal. Calcd for C$_{25}$H$_{34}$N$_4$O$_8$: C, 57.90; H, 6.61; N, 10.80. Found: C, 57.83; H, 6.68; N, 10.65.

10   **D.   N-(N-Boc-2-aminoethyl)-N-[(1-thyminyl)acetyl]-L-serine(OBn) (39)**

The substrate **39C** (0.52g, 1 mmol) was dissolved in THF (10ml) and cooled to 0°C in an ice bath. To this cold stirred solution NaOH (1N, 5ml, 5 mmol) was added all at once. The reaction mixture was stirred at 0°C for 1h and evaporated to dryness. The residue was dissolved in water (10ml) and the pH was adjusted to 3-4 with solid citric acid. The

15   aqueous solution was extracted with EtOAc (3x30ml). The organic extract was washed with brine, dried and evaporated to dryness to give a foam. The residue was dried over solid NaOH and checked by HNMR. Yield 0.25g (49%). ^{1}HNMR (DMSO-d$_6$) d 1.38 (s, 9H, t-Butyl), 1.72 (s, 3H, CH_3), 3.00 to 4.00 (m, 4H, 2CH_2), 4.40 (m, 5H, CH, CH_2, & OCH_2), 6.80 (t, 1H, NH), 7.22 (m, 6H, C$_6H$ & ArH) and 11.25 (m, 1H, COOH).

20

EXAMPLE 40

N-(N-Boc-2-Aminoethyl)-N-(2, 2, 2-trichloro-1, 1-dimethyl-1-ethyoxycarbonyl)-L-serine[(α-methoxy-α-trifluoromethyl-α-phenyl)-O-acetate] methyl ester (40)

A.   N-(N-Boc-2-aminoethyl)-L-serine(OH) methyl ester (40a)

25   The substrate **39b** (0.7g, 2 mmol) was dissolved in methanol (20ml) and treated with Pd/C (10%, 0.2g). The reaction mixture was hydrogenated for 12h at 50psi H$_2$. The catalyst was filtered and washed with MeOH (20ml). The combined filtrate was evaporated to dryness. The residue was purified by flash column chromatography over silica gel using CH$_2$Cl$_2$/MeOH as the eluent. The pure fractions were collected and evaporated to give 0.9g

30   (95%) of the product as a foam. ^{1}HNMR (CDCl$_3$) d 1.42 (s, 9H, t-Butyl), 2.70 (m, 1H,

CH$_2$), 2.88 (m, 1H, CH$_2$), 3.20 (m, 2H, CH$_2$), 3.45 (m, 1H), 3.62 to 3.90 (m, 6H, CH$_2$, OCH$_3$), 5.15 (bs, 1H, NH).

B.   N-(2-Boc-Aminoethyl)-N-(2,   2,   2-trichloro-1,   1-dimethyl-1-ethyoxycarbonyl)-L-serine(OH) methyl ester (40b)

5   The substrate **40a** (2.62g, 10 mmol) was dissolved in ether (30ml) and mixed with 2N NaHCO$_3$ solution (20ml). To this stirred solution was added 1,1-dimethyl-2,2,2-trichloroethyl chloroformate (2.39g, 10 mmol, dissolved in 20ml of ether) slowly during 15min period at room temperature. After the addition of TecBocCl, the reaction was stirred for 12h and concentrated to dryness. The residue was suspended in water (50ml) and

10   extracted in CH$_2$Cl$_2$ (2x75ml). The organic extract was washed water (50ml) and brine(50ml). The CH$_2$Cl$_2$ layer was dried over anhydrous Na$_2$SO$_4$ and evaporated to dryness. The residue was purified by flash column chromatography over silica gel using CH$_2$Cl$_2$/acetone as the eluent. The pure fractions were collected and evaporated to give 3.9g(85%) of an oily product. ^{1}HNMR (CDCl$_3$) d 1.42 (s, 9H, t-Butyl), 1.90 (s, 6H, 2CH$_3$),

15   3.35 (m, 2H, CH$_2$), 3.50 (m, 2H, CH$_2$), 3.75 (s, 3H, OCH$_3$), 3.90 to 4.30 (m, 3H, OH & CH$_2$), 4.45 (m, 1H, CH), 5.06 (m, 1H, NH).

C.   N-(N-Boc-2-Aminoethyl)-N-(2,   2,   2-trichloro-1,   1-dimethyl-1-ethyoxycarbonyl)-L-serine[(α-methoxy-α-trifluoromethyl-α-phenyl)-O-acetate] methyl ester (40)

20   The substrate **40b** (0.3g, 0.65 mmol) was dissolved in dry CH$_2$Cl$_2$ (30ml) and cooled to 0°C under argon. To this cold stirred solution was added dry pyridine (1ml) followed by a-methoxy-a-trifluoromethyl-a-phenyl acetyl chloride (0.18g, 0.7 mmol). After addition of the acid chloride, the reaction was allowed to stir for 12h. The reaction mixture was diluted with CH$_2$Cl$_2$ (75ml) and washed with 5%NaHCO$_3$ (100ml), water (50ml) and brine(50ml).

25   The CH$_2$Cl$_2$ layer was dried over anhydrous Na$_2$SO$_4$ and evaporated to dryness. The residue was loaded on a column loaded with silica gel using CH$_2$Cl$_2$. The excess Moser's acid was eluted first with CH$_2$Cl$_2$. Then the column was eluted with CH$_2$Cl$_2$/acetone (8:2) to give the product. The product was checked by ^{19}F for chiral purity. The ^{19}F showed two peaks at 20°C for the rotamer around the amide bond. But the two peaks became a single peak at

30   80°C. No raceimization was observed. ^{1}H NMR (CDCl$_3$) d 1.42 (s, 9H, t-Butyl), 1.88 (s,

6H, 2CH_3), 3.00 (m, 2H, CH_2), 3.50 (s, 3H, OCH_3), 3.75 (s, 3H, OCH_3), 4.28 (m, 1H, CH),

4.80 (m, 2H, CH_2), 5.10 (m, 1H, NH) and 7.45 (m, 5H, ArH). ^{19}F NMR (DMSO-d$_6$) ppm -

72.699 & -72.78(20°C), -71.15(80°C) with TFA (-76.53ppm) as internal standard.

5   **EXAMPLE 41**

N-(N-Boc-2-Aminoethyl)-N-(2, 2, 2-trichloro-1, 1-dimethyl-1-ethyoxycarbonyl)-L-serine[(a-methoxy-a-trifluoromethyl-a-phenyl)-O-acetate] methyl ester (41)

A.   **N-(N-Boc-2-aminoethyl)-D-serine(O-benzyl) methyl ester (41a)**

The titled compound was prepared by following the method B Example **39**,

10   described for the preparation of L-isomer **39b**. The reactants used: Boc-aminoacetaldehyde (8.27g, 52 mmol), D-serine(0Bn) methyl ester (11.0g, 52.63 mmol), NaOAc (7.07g, 52 mmol), Pd/C (2g) and dry MeOH (100ml). The crude product was purified by flash chromatography using CH$_2$Cl$_2$/ethyl acetate as the eluent. Yield 12g

(66%). ^{1}HNMR (CDCl$_3$) δ 1.44 (s, 9H, t-Butyl), 1.95 (bs, 1H, NH), 2.60 (m, 1H, CH_2),

15   2.80 (m, 1H, CH_2), 3.18 (m, 2H, CH_2), 3.44 (t, 1H), 3.68 (m, 2H, CH_2), 3.72 (s, 3H, OCH_3), 4.50 (s, 2H, OCH_2), 5.06 (bs, 1H, NH) and 7.26 (m, 5H, ArH). Anal. Calcd for C$_{18}$H$_{28}$N$_2$O$_5$: C, 61.34; H, 8.01; N, 7.95. Found: C, 61.31; H, 8.12; N, 7.85.

B.   **N-(2-Boc-Aminoethyl)-D-serine methyl ester (41b)**

The titled compound was prepared by following the procedure described for the

20   preparation of L-isomer **40a**, Example Y. The reactants used were: The substrate **41a**, (0.71g, 2 mmol), Pd/C (10%, 0.2g) and dry MeOH (20ml). The crude product was purified by flash chromatography using CH$_2$Cl$_2$/MeOH as the eluent. Yield 0.9g (95%). ^{1}HNMR (CDCl$_3$) δ 1.42 (s, 9H, t-Butyl), 2.70 (m, 1H, CH_2), 2.88 (m, 1H, CH_2), 3.20 (m, 2H, CH_2), 3.45 (m, 1H), 3.62 to 3.90 (m, 6H, CH_2, OCH_3), 5.15 (bs, 1H, NH).

25   C.   **N-(N-Boc-2-aminoethyl)-N-(2, 2, 2-trichloro-1, 1-dimethyl-1-ethyoxycarbonyl)-D-serine(OH) methyl ester (41c)**

The titled compound was prepared by following the procedure described for the preparation of L-isomer **40b**. The reactants used were: The substrate **41b**, (0.5g, 1.91 mmol), 1,1-dimethyl-2,2,2-trichloroethyl chloroforamate (0.72g, 3 mmol), 1N NaHCO$_3$

30   (5ml, 5 mmol) and ether (10ml). The crude product was purified by flash

chromatography using CH_2Cl_2/acetone as the eluent. Yield 0.5g (56%). ${}^1HNMR$ (CDCl$_3$) δ 1.42 (s, 9H, t-Butyl), 1.90 (s, 6H, 2CH_3), 3.35 (m, 2H, CH_2), 3.50 (m, 2H, CH_2), 3.75 (s, 3H, OCH_3), 3.90 to 4.30 (m, 3H, OH & CH_2), 4.45 (m, 1H, CH), 5.06 (m, 1H, NH).

5   **D.   N-(N-Boc-2-Aminoethyl)-N-(2, 2, 2-trichloro-1, 1-dimethyl-1-ethyoxycarbonyl)-L-serine[(α-methoxy-α-trifluor omethyl-α-phenyl)-O-acetate] methyl ester (41d)**

        The substrate **41c** 15 (0.3g, 0.65 mmol) was dissolved in dry CH_2Cl_2 (30ml) and cooled to 0°C under argon. To this cold stirred solution was added dry pyridine (1ml)
10   followed by α-methoxy-α-trifluoromethyl-α-phenyl acetic acid chloride (0.18g, 0.7 mmol). After the addition of acid chloride, the reaction was allowed to stir for 12h. The reaction mixture was diluted with CH_2Cl_2 (75ml) and washed with 5%NaHCO$_3$ (100ml), water (50ml) and brine (50ml). The CH_2Cl_2 layer was dried over anhydrous Na$_2$SO$_4$ and evaporated to dryness. The residue was loaded on a column silica gel using
15   CH_2Cl_2. The excess Moser's acid was eluted first with CH_2Cl_2. Then the column was eluted with CH_2Cl_2/acetone (8:2) to give the product. The product was checked by${}^{19}F$ for chiral purity. The ${}^{19}F$ showed two peaks at 20°C for rotamer around the amide bond. But the two peaks became single peak at 80°C. No raceimization was observed. The L-isomer (**40**) and D-isomer (**41d**) were mixed and checked by ${}^{19}F$ NMR. The mixture
20   showed a triplet (ppm, -70.772, -70.856 & -70.947) at 20°C, but at 80°C a triplet became a doublet (ppm, -70.679 & -70.758). This accounts for the DL mixture and rotamer at 20°C. On the other hand at 80°C they exist only as the DL isomer. ${}^1H$ NMR (DMSO-d$_6$) δ 1.38 (s, 9H, t-Butyl), 1.80 (m, 6H, 2CH_3), 3.00 (m, 2H, CH_2), 3.32 (m, 2H, CH_2), 3.42 (s, 3H, OCH_3), 3.64 (s, 3H, OCH_3), 4.76 (m, 3H, CH & CH_2), 6.64 (m, 1H, NH)
25   and 7.45 (s, 5H, ArH). ${}^{19}F$ NMR (DMSO-d$_6$) ppm -70.86 & -70.96(20°C), -70.75(80°C) with TFA (-76.53ppm) as internal standard.

        E.   N-(N-Boc-2-aminoethyl)-N-[(1-thyminyl)acetyl]-D-serine(OBn) methyl ester (41e)

        The titled compound was prepared by following the procedure described for the
30   preparation of L-isomer **39c**. The reactants used were: The substrate **41a** (10.3g, 29.3

mmol), thymin-1-ylacetic acid (6.07g, 33 mmol), DhbtOH (5.4g, 33 mmol), EDC (6.3g,

33 mmol), dry CH_2Cl_2 (150ml) and dry DMF (50ml). The crude product was purified

by flash chromatography using CH_2Cl_2/acetone as the eluent. Yield 13.2g (87%).

^{1}HNMR (CDCl$_3$) δ 1.42 (s, 9H, t-Butyl), 1.90 (s, 3H, CH_3), 3.32 (m, 2H, CH_2), 3.55 (m,

2H, CH_2), 3.72 (s, 3H, OCH_3), 4.00 (m, 2H, CH_2), 4.32 (m, 1H, CH), 4.50 (s, 2H,

OCH_2), 4.52 (m, 2H, CH_2), 5.46 (t, 1H, NH), 6.85 (s, 1H, C$_6H$), 7.30 (m, 5H, ArH) and

8.8 (s, 1H, NH). Anal. Calcd for $C_{25}H_{34}N_4O_8$: C, 57.90; H, 6.61; N, 10.80. Found: C,

57.82; H, 6.67; N, 10.67.

EXAMPLE 42

N-(N-Boc-2-aminoethyl)-N-[(1-thyminyl)acetyl]-L(or D)-serine methyl ester (42)

Compound **39** (0.5g) is dissolved in ethanol and palladium on carbon (50mg) is

added. The mixture is shaken on a Parr hydrogenation apparatus for 24h. The reaction

mixture is filtered and concentrated. The residue is purified by flash column

chromatography to give the title compound.

EXAMPLE 43

N-(N-Boc-2-aminoethyl)-N-[(1-thyminyl)acetyl]-L(or D)-serine methyl ester (43)

Compound **42** is converted to the title compound using the procedures of

Example **37.**

EXAMPLE 44

N-(N-Boc-2-aminoethyl)-N-[(1-thyminyl)acetyl]-L-serine methyl ester (44)

Compound **43** is converted to the title compound using the procedures of

Example **38.**

EXAMPLE 45

N-Methyl PNA Monomers, General Procedures for Synthesis and Oligomerization

1.   Method A

A.   N-Methyl-1-amino-2,3-propandiol (45A)

Methylamine (172ml of a 40% solution in water, 2 mol) was cooled to 0 °C and

2,3-epoxy-1-propanol (25g, 0.34mol) was added at a rate to maintain the temperature at

or below 10 °C. The mixture was stirred for 3 hours at 0 °C. Excess methylamine and

5   water was evaporated in *vacuo* and the product was purified by distillation at 103-105

°C, at 0.5 mm Hg, to give 25.6g (76%) of the title compound.

B.   N-Boc-N-methyl-1-amino-2,3-propandiol (45B)

To a solution of N-methyl-1-amino-2,3-propandiol (21g, 0.2mol) in water

(340ml) maintained at 0 °C was added Boc-anhydride (52.3g, 0.24mol). The mixture

10   was allowed to equilibrate to room temperature. The pH was adjusted to and maintained

at 10.5 with 4N NaOH. NaOH (2 eq.) is added and the reaction mixture was kept at

room temperature under an atmosphere of nitrogen overnight. The reaction mixture is

cooled to 0 °C and the pH is adjusted to 2.5 with 4N HCl. The acidic mixture is

extracted with ethylacetate (6x100ml). The ethylacetate extracts were combined,

15   washed with half-saturated $KHSO_4$ (3x150ml) and saturated brine (1x150ml), dried over

$MgSO_4$ and evaporated to dryness. The title compound was isolated as an oil (bp 110-

112 °C at 0.5 mm Hg). The yield of the title compound was 31.3g (81%).

C.   N-Boc-N-methyl-2-amino-acetaldehyde (45C)

To a solution of N-Boc-N-methyl-1-amino-2,3-propandiol   (20g, .097 mol) in

20   water (100ml) was added KIO_4 (24.68g, .108 mol). The reaction mixture was stirred for

2.5 hr under an atmosphere of nitrogen and then filtered. The filtrate was extracted with

chloroform (5x50ml). The resulting chloroform extracts are dried over $MgSO_4$, filtered,

and evaporated to dryness. The title compound 13.26g (79%) was isolated as a clear oil

(bp 76-80 °C at 0.5 mm Hg).

25   ### D.   N-[(N-Boc-N-methyl)-2-aminoethyl]glycine ethyl ester (45D)

Glycine ethyl ester hydrochloride (0.58 mol) was dissolved in absolute ethanol

(100ml) and added dropwise to a solution of N-Boc-N-methyl-2-amino-acetaldehyde

(10g, .058 mol) and NaOAc (9.32g, .114 mol) dissolved in absolute ethanol (130ml) at 0

°C. This solution was hydrogenated using palladium on carbon (1.65g) and hydrogen

30   (1eq) overnight. The reaction mixture was filtered and water (80ml) was added. The

pH was adjusted to 8 with 2N NaOH and extracted with dichloromethane (5x60ml).
The organic phase was dried over $MgSO_4$ and evaporated to dryness. The residue was
purified by kugelrohr distillation (100 °C, 0.5 mm Hg) to give 10.6g (74.3%) of the title
compound as a clear oil.

5   E.   **General Procedure for synthesis and deprotection of the Monomers**
(A, C, G, T)

The monomers containing the four natural bases attached via an acetyl linker
were synthesized using N-Boc-N-methyl-1-aminoethylglycine ethyl ester (X). To a
solution of N-Boc-N-methyl-1-aminoethylglycine ethyl ester (X, 1.82g, 7mmol) in dry

10   DMF (30ml) was added DHBT-OH (1.26g, 7.7 mmol) and the carboxymethyl derivative
of the base. Dichloromethane (30ml) was added and the solution was cooled to 0 °C.
DCC (1.73g, 0.84 mmol, 1.2 eq) was added and the mixture was stirred for 1 hr. The
reaction mixture was allowed to warm to room temperature and stirred for 3 hr and then
filtered.

15   The protected A monomer, N-[(N-Boc-N-methyl)-2-aminoethyl]-N-[(9-
adeninyl)acetyl]glycine ethyl ester, was synthesized using the above procedures. The
reaction mixture was evaporated, redissolved in dichloromethane (300ml), washed with
half-saturated aqueous $NaHCO_3$ (3x100ml), half-saturated aqueous $KHSO_4$, (2x100ml)
and saturated brine (1x100ml). The organic phase was dried over $MgSO_4$ and

20   evaporated to dryness. Absolute ethanol (60ml) and water (30ml) was added to the
residue and the mixture was stirred overnight. The resulting precipitate was filtered and
dried to afford the protected A monomer.

The protected A monomer is dissolved in THF and cooled to 0 °C. LiOH (25ml)
was added and the reaction mixture was stirred for 30 min. The pH was adjusted to 1

25   with HCl (2N). The resulting precipitate was filtered and dried to afford N-[(N-Boc-N-
methyl)-2-aminoethyl]-N-[(9-adeninyl)acetyl]glycine.

The protected C monomer, N-[(N-Boc-N-methyl)-2-aminoethyl]-N-[(1-
cytosinyl)acetyl]glycine ethyl ester, was synthesized using the above procedures. The
reaction mixture was evaporated to dryness. Ether (50ml) was added to the residue and

30   the mixture was stirred for 2 hr and filtered. This was repeated two times and then

followed by addition of half-saturated aqueous NaHCO₃, filtered and dried. The
resulting residue was dissolved in boiling dioxane (60ml) and precipitated with water
(60ml), filtered and dried. The precipitate was purified by silica gel column
chromatography using methanol/dichloromethane 5/95 to afford the protected C

5   monomer.

The protected C monomer is dissolved in THF and cooled to 0 °C. LiOH (60ml)
was added and the reaction mixture was stirred for 30 min. The pH was adjusted to 2
with HCl (2N). The mixture was extracted with dichloromethane and the organic phase
dried over MgSO₄ and evaporated to afford .72g (27%) N-[(N-Boc-N-methyl)-2-

10   aminoethyl]-N-[(1-cytosinyl)acetyl]glycine (mp 181-184 °C).

The protected G monomer, N-[(N-Boc-N-methyl)-2-aminoethyl]-N-[(9-
guaninyl)acetyl]glycine ethyl ester, was synthesized using the above procedures. The
reaction mixture was evaporated, redissolved in dichloromethane (80ml), washed with
half-saturated aqueous KHSO₄, (3x40ml), dried over MgSO₄, and evaporated to dryness.

15   The precipitate was recrystallized in ethylacetate and dried to give the protected G
monomer.

The protected G monomer is dissolved in methanol (40ml) NaOH (40ml, 2N)
was added. The mixture was stirred at room temperature for 1 hr and then cooled to 0
°C. The pH was adjusted to 2 with HCl (2N). The resulting precipitate was filtered,

20   dried and recrystalized from absolute ethanol to afford 1.27g (43%) of N-[(N-Boc-N-
methyl)-2-aminoethyl]-N-[(9-guaninyl)acetyl]glycine (mp 189-192 °C).

The protected T monomer, N-[(N-Boc-N-methyl)-2-aminoethyl]-N-[(1-
thyminyl)acetyl]glycine ethyl ester, was synthesized using the above procedures.
Dichloromethane (60ml) was added to the reaction mixture and the resulting mixture

25   was washed with half-saturated NaHCO₃ (3x30ml), half-saturated aqueous KHSO₄
(2x30ml), and saturated brine (1x30ml). The organic phase was dried over MgSO₄ and
evaporated. The residue was redissolved in dichloromethane, cooled to 0 °C and
precipitated by slow addition of petroleum ether with vigorous stirring. Filtration of the
mixture gave the protected T monomer as a white solid.

30   The protected T monomer is dissolved in methanol (45ml) and cooled to 0 °C.

NaOH (45ml, 2N) was added and the mixture was stirred at room temperature for 2 hrs. The pH was adjusted to 2 with HCl (2N) and the organic phase was extracted with ethylacetate. The organic phase was dried over $MgSO_4$ and evaporated to dryness to afford 1.45g (52%) of N-[(N-Boc-N-methyl)-2-aminoethyl]-N-[(1-thyminyl)acetyl]-
glycine (mp 114-118 °C).

F.   General Procedures for Oligomerization

The prepared monomers were used for oligomerization by solid phase peptide synthesis on MBHA-resin.

Using the above procedures the following oligomers were prepared:

H-GT*AGAT*CACT*Lys-CONH$_2$	(SEQ ID NO:33)
H-Gly-G*T*A*G*A*T*C*A*C*T*-Lys-CONH$_2$	(SEQ ID NO:34)
H-TTTTTT*TTTT-Lys-CONH$_2$	(SEQ ID NO:35)
H-TTTT*TT*TTT-Lys-CONH$_2$	(SEQ ID NO:36)
H-TTTT*TT*TT*TT-Lys-CONH$_2$	(SEQ ID NO:37)
H-Gly-T*T*T*T*T*T*T*T*T*T*-Lys-CONH$_2$	(SEQ ID NO:38)

wherein a * denotes a monomer functionalized to have an N-methyl group on the 2-aminoethyl portion of the monomer.

2.   Method B

A.   N-Boc sarcosylglycine methyl ester

To a suspension of Boc-sarcosine (9.0 g, 048 mol) in 70 ml of CH_2Cl_2 at -20°C was added isobutyl chlorofomate (7.1g, 0.052mol) and N-Methylmorpholine (4.8 g, 0.047mol). After 2 min glycine methyl ester, hydrochloride (7.19g, 0.057mol) and N-methylmorpholine (5.76g, 0.057 mol) in CH_2Cl_2 (60 ml) was added. The reaction was stirred at -10°C for 1 h and allowed to warm to room temperature. After 4h at room temperature, the reaction was filtered and washed with salt. $NaHCO_3$ (100ml), 1N $NaHCO_3$ 100 ml, twice with 1N $KHSO_4$ (100 ml), water (100 ml) and brine (70 ml). The organic phase was dried with Na_2SO_4 and evaporated. The yield of Boc sarcosyl-
glycine methyl ester was 89%.

B. N-[(N-Boc)(N-methyl)(1-thiocarbonyl)-2-aminoethyl]-glycine methyl ester

A suspension of the N-Boc-sarcosylglycine methyl ester (10.0 g, 0.041 mol) and Lawessons reagent (9.87 g, 0.024mol) in toluene (90 ml), was heated to 80°C for 1h and then evaporated to dryness. The product was purified by column chromatography (CH$_2$Cl$_2$/MeOH 95:5) on silica gel to give 63% of the title compound.

C. N-[(N-Boc)(N-methyl)-2-aminoethyl]-glycine methyl ester

To a suspension of the N-[(N-Boc)(N-methyl)(1-thiocarbonyl)-2-aminoethyl]-glycine methyl ester (2.67 g, 0.0097 mol) and NiCl$_2$6H$_2$O (18.39 g, 0.077 mol) in THF:MeOH 1:1 (100ml) at -20°C, NaBH$_4$ (8.78g, 0.23 mol) was slowly added so that the temperature did not exceed 0°C. After addition of the NaBH$_4$, the reaction was allowed to go to room temperature. The reaction mixture was filtered several times through celite and evaporated to dryness. The product was isolated by column chromatography on silica gel with CH$_2$CL$_2$/MeOH 90:10 eluent. The yield of the title compound was 60%.

D. N-[(N-Boc)(N-methyl)-2-aminoethyl]-N-[(1-thyminyl)acetyl] glycine methyl ester

To a suspension of N-[(N-Boc)(N-methyl)-2-aminoethyl]-glycine methyl ester (0.9 g, 0.0037 mol) and DHBT-OH (0.65 g, 0.004 mol) in CH$_2$Cl$_2$ 10 ml was added N-1-carboxymethylthymine (0.74 g, 0.04 mol) dissolved in DMF (6ml). The solution was cooled in an ice bath and DCC (0.905, 0.049 mol) was added. The reaction mixture was stirred for 1 hour and the ice bath was removed. The reaction mixture was left for 16 hours at room temperature. The reaction mixture was filtered and washed twice with 1N KHSO$_4$ (15 ml) and three times with NaHCO$_3$ (15 ml), brine (10 ml) and water (10 ml) and dried with Na$_2$SO$_4$. The product was purified by column chromatography on silica gel with CH$_2$Cl$_2$/MeOH 95:5 eluent. The yield of the title compound was 55%.

E.   N-[(N-Boc)(N-methyl)-2-aminoethyl]-N-[(1-thyminyl)acetyl] glycine

N-[(N-Boc)(N-methyl)-2-aminoethyl]-N-[(1-thyminyl)acetyl] glycine methyl ester was dissolved in 1N NaOH in MeOH/H$_2$O 1:1 for 2 hours. The pH was adjusted to 2 with 2N HCl and extracted with ethyl acetate (50 ml). The organic phase was dried

with Na_2SO_4 and evaporated to dryness. The yield of the title compound was 77%.

F. Oligomerization of N-methyl monomers

MBHA resin was adjusted to a loading of 0.1 mmol/mg by applying N-Boc N'-2-chloro-Z-lysine and DIC in CH_2Cl_2/DMF 1:1 24 hours. Coupling of the monomers including

5   the N-methyl derivative follows the normal protocol for coupling of PNA monomers to the solid support (see Example 26). Coupling of the monomer following the N-methylated monomer follows this protocol: 3 eq. of the monomer and 3 eq. of PyBorP was dissolved in CH_2Cl_2/DMF and added to the solid phase. Diisoproylethylamine (9 eq.) was added. The reaction mixture was left for 24 hours. The reaction mixture was

10   washed twice with DMF for a few seconds and once with DMF for two minutes. Then it was washed four times with CH_2Cl_2. This was repeated four times and the standard protocol for PNA oligomerization was then followed again.

15   **EXAMPLE 46**

Alternative Synthetic Route for Production of Functionalized PNA Monomers

        A.   **(S)-3-Amino-2-oxetanone p-toluenesulfonate (46A)**

        Boc serine is treated with Ph_3P, DEAD, and TFA , followed by TsOH to give the title compound.

20   **B.**   **[(2-N-Tosyl)-3-N-Boc]propionic acid (46B)**

        Compound **46A** is treated with Boc_2NH to give the title compound.

        C.   **Ethyl (2-N-phthaloyl)-(3-N-Boc)-2,3-diaminopropionate (46C)**

        Compound **46B** is treated with ethoxycarbonylphthalimide followed by ethanol, DCC and HOBT to give the title compound.

25   **D.**   **Ethyl-(2-N-phthaloyl)-2,3-diaminopropionate (46D), ethyl-(3-N-Boc)-2,3-diaminopropionate (46E)**

        Compound **46C** is either deprotected at the C-3 Boc group to give the amino group by treatment with TFA/dichloromethane to give Compound **46D**, or deprotected at the C-2 amino group by treatment with hydrazine to give **46E**.

30   The compounds ethyl-(2-N-phthaloyl)-2,3-diaminopropionate and ethyl-(3-N-

Boc)-2,3-diaminopropionate are used directly in the syntheses of functionalized PNA

backbones as described in Examples **30-38**.

EXAMPLE 47

5 **Incorporation of Biotin into an Aminoethyl Glycine-PNA Oligomer H-**

TTC(Biotin)-TT-Lys-NH₂ (SEQ ID NO:39)

The protected aminoethyl glycine-PNA oligomer is assembled on a Boc-

Lys(ClZ) modified MBHA resin with a substitution of approximately 0.10 mmol/g. The

synthesis is initiated on 100 mg (dry weight) of t-Boc-Lys(ClZ)-MBHA resin,

10 preswollen in dichloromethane. The aminoethyl glycine-PNA-oligomer is synthesized

as per the procedures of Example **26**. In step (3) an aeg-cytosine monomer from

Examples **10** thru **17** which has been further derivatized following the procedures of

Sproat B.S., *et al., Nucleic Acids Research*, **1989**, *17*, 3373-3386, to include an N-4 (5-

trifluoroacetylaminopentyl) protected linking moiety. The procedure of Sproat is

15 modified for use with a PNA monomer by simply using an aminoethyl glycine cytosine

monomer having the N-4 exocyclic amino group deprotected and the amino and the

carboxyl group of the aminoethyl glycine protected. The aminoethyl glycine-PNA was

cleaved from the resin as per the procedures of Example **28**. Yield: 5.8 mg (purity

90%), purified by RP-HPLC, (μBondapak C18). MS(FAB+) *m/z*:(found/calcd)

20 2702/2701

EXAMPLE 48

N-Terminal Conjugation of Biotin to a PNA

The N-terminal Boc-blocked PNA containing resin is measured out to typically

25 provide 0.1-1.0 millimoles of PNA. This weight of resin is suspended in a 1:1 (v:v)

dichloromethane:dimethylformamide solution (5mL/100mg of resin) and allowed to

swell for a period of time if desired. The solvent is then removed by filtration and the

resin resuspended in trifluoroacetic acid (3mL/100mg of resin) and shaken for 2

minutes. The trifluoroacetic acid is removed by filtration and this is repeated one time.

30 The resulting resin is resuspended in pyridine (1mL/10μmoles of resin) and vacuum

filtered to remove the pyridine. This is repeated. This washing step is repeated twice. The resin is suspended in 1:1 (v:v) pyridine:dimethylformamide and to this suspension is added the biotin (2-10 molar equivalents), TBTU (1.9-9.9 molar equivalents), and di-isopropylethlamine (5-20 molar equivalents) such that the final concentration of PNA

5   monomer is 0.2M. The suspension is shaken for 15-60 minutes and the spent coupling solution is removed by filtration. The resin is then washed three times with 1:1 (v:v) dichloromethane:dimethylformamide solution (5mL/100mg of resin) giving the N-terminal biotin-attached PNA. This can be cleaved from the resin as described in Example **28**. Starting with BocGly-GCAT-CO-Merrifield resin one obtains Biotin-Gly-

10   GCAT-COOH and starting with BocGly-GCAT-Lys-CO-Merrifield resin one gets Biotin-Gly-GCAT-COOH.

EXAMPLE 49

N-Terminal Conjugation of Pteroic Acid to a PNA

15   An N-Boc-O-Benzyl-Blu-blocked PNA containing resin is measured out to typically provide 0.1-1.0 millimoles of PNA. This weight of resin is suspended in a 1:1 (v:v) dichloromethane:dimethylformamide solution (5mL/100mg of resin) and allowed to swell for a period of time if desired. The solvent is then removed by filtration and the resin resuspended in trifluoroacetic acid (1mL/100mg of resin) and shaken for 2

20   minutes. The trifluoroacetic acid is removed by filtration and this is repeated one time. The resulting resin is resuspended in pyridine solution (1mL/1gm of resin) and vacuum filtered to remove the pyridine. This is repeated. This is followed by resuspension followed by filtration (designated "washing") using 1:1 (v:v) dichloromethane:dimethylformamide solution (5mL/1gm of resin). This washing step is

25   repeated twice. The resin is suspended in 1:1 (v:v) pyridine:dimethylformamide and to this suspension is added the N-Boc pteroic acid (prepared from pteroic acid, Aldrich Chem Co., 2-10 molar equivalents), TBTU (1.9-9.9 molar equivalents), and di-isopropylethylamine (5-20 molar equivalents) such that the final concentration of PNA monomer is 0.2m. The suspension is shaken for 15-60 minutes and the spent coupling

30   solution is removed by filtration. The resin is then washed three times with 1:1 (v:v)

dichloromethane:dimethylformamide solution (5mL/100mg of resin) giving the N-terminal folic acid-attached PNA. This can be cleaved from the resin as described in Example **28**. Starting with N-Box-O-Benzyl-Glu-GCAT-CO-Merrifield resin one obtains Boc-folate-GCAT-COOH and starting with N-Boc-O-Benzyl-Glu-GCAT-Lys-

5 COMerrifield resin one gets N-Boc-folate-GCAT-COOH.

EXAMPLE 50

Coupling to a Free COOH of a PNA or PNA Derivative

A PNA or PNA derivative having a single free COOH such as CH_3CONH-

10 GCAT-COOH is dissolved in tetrahydrofuran:water to provide a solution that is 0.1 M in PNA and to this is added 1-(3-dimethylaminopropyl)-3-ethylcarbodimide hydrochloride [EDCI] and Rhodamine 123 hydrate to give a solution which is 0.1-1.0 molar in both EDCI and Rhodamine. The solution is stirred for 0.1-2 hours, then evaporated to a solid and purified by preparative HPLC as described in Example **29**.

15 By this method one can prepare the following compounds.

Rhodamine = Rhod

CH₃CONH-GCAT-CONHRhod (SEQ ID NO:40)

CH₃CONHY-GCAT-Asp(Rhod)-CONH₂ (SEQ ID NO:41)

Chol-GlyGCAT-Asp(Rhod-CONH₂ (SEQ ID NO:42)

20 Chol-GCAT-Asp(Rhod)-CONH₂ (SEQ ID NO:43)

RhodNHOC(CH₂)₄CONH-GCAT-Lys-CONH₂ (SEQ ID NO:44)

RhodNHOC(CH₂)₄CONH-GCAT-CONH₂ (SEQ ID NO:45)

H₂N-GCAT-CONHRhod (SEQ ID NO:46)

H₂N-Gly-GCAT-CONRhod (SEQ ID NO:47)

25 H₂N-GCAT-Lys-CONHRhod (SEQ ID NO:48)

H₂N-Gly-GCAT-Lys-CONHRhod (SEQ ID NO:49)

Fluor-GCAT-CONHRhod (SEQ ID NO:50)

Fluor-GlyGCAT-CONHRhod (SEQ ID NO:51)

Fluor-GCAT-Gly-CONHRhod (SEQ ID NO:52)

30 CH₃CONH-GCAT-Lys(Fluor)-CONRhod (SEQ ID NO:53)

Chol-GlyGCAT-Lys(Fluor)-CONHRhod (SEQ ID NO:54)

Chol-GCAT-Lys(Fluor)-CONRhod (SEQ ID NO:55)

RhodNHOC(CH$_2$)$_4$ACONH-GCAT-Lys(Fluor)-CONH2 (SEQ ID NO:56)

Fluor-GlyGCAT-Lys(Fluor)-CONHRhod (SEQ ID NO:57)

5   Fluor-GCAT-Lys(Fluor)-CONHRhod (SEQ ID NO:58)

EXAMPLE 51

6-S-Tritylthiohexylbromide (51), 6-S-tritylthiohexanoic acid (51A), 6-S-tritylthiohexylamine (51B), 6-S-tritylthiohexylmercaptan (51C)

10   To a solution of triphenylmethanethiol (Fluka; 69g, 250 mmol) in 500 mL 95%

ethanol (EtOH) was added 11 grams of sodium hydroxide dissolved in 75 mL of water

(275 mmol). After stirring for about 15 minutes under an atmosphere of argon, using an

addition funnel, 1,6-dibromohexane (91.5 g, 375 mmol, 58 mL) dissolved in 100 mL of

95% EtOH was added dropwise over a period of 1 hour with vigorous stirring. After

15   about 15 minutes of stirring, a brown white solid separates out from the reaction

mixture. After stirring for an additional 4 hours, the reaction mixture was filtered. The

filtrate was evaporated under high vacuum and the oily residue was combined with the

filtered residue and dissolved in 500mL CH$_2$Cl$_2$, and filtered again. The filtrate was

washed once with water (200 mL) and once with saturated NaCl solution. After drying

20   the CH$_2$Cl$_2$ layer over MgSO$_4$, it was concentrated to 200 mL in volume. About 200 mL

of hexane was added and the solution was left in the freezer. Three crops of cream

white product was isolated to give 81g of 6-S-Tritylthiohexylbromide (51), mp 91-92

°C.

Portions of the product are independently treated with sodium cyanide followed

25   by hydrolysis to give the corresponding acid, 6-S-tritylthiohexanoic acid (Compound

51A), with lithium azide followed by triphenylphosphine reduction to give the

corresponding amine, 6-S-tritylthiohexylamine (Compound **51B**), and with sodium

hydrogen sulfide to give the corresponding thiol, 6-S-tritylthiohexylmercaptan

(Compound **51C**).

30

EXAMPLE 52

N-[(N-Boc)(1-(6-S-tritylhexyloxymethyl))2-aminoethyl]-N-[(1-thyminly)acetyl]glycine ethyl ester (52A) and N-[(N-Boc)(2-(6-S-tritylhexyloxymethyl))2-aminoethyl]-N-[(1-thyminly)acetyl]glycine ethyl ester

5   (52B)

The free carboxyl group of compound **35** or **36** is protected as the ethyl ester according to standard procedures (*see, e.g.*, Greene and Wuts, Protective Groups in Organic Synthesis, 2d edition, John Wiley & Sons, New York, 1991). The resulting ethyl aminoethylglycinate is alkylated with *S*-trityl-6-mercaptohexylbromide in the

10   presence of DMF and sodium hydride to yield the title compounds.

EXAMPLE 53

N-[((N-Boc-)(1-{(6-thio)hexyloxymethyl})2-aminoethyl]-N-[(1-thyminly)acetyl]glycine ethyl ester (Compound 53A) and N-[((N-Boc-)(2-{(6-

15   thio)hexyloxymethyl})2-aminoethyl]-N-[(1-thyminly)acetyl]glycine ethyl ester

(Compound 53B)

Compound **52A** or **52B** (0.19 mmol) is dissolved in 4 mL chloroform. Silver Nitrate (8 mM) in EtOH (12 mL) is added and the reaction mixture is stirred for 45 minutes. Dithiothreitol (0.35 M) in chloroform (3 mL) is added and the reaction is stirred overnight.

20   The white precipitate is filtered off and the solvent removed *in vacuo*. The residue is dissolved in dichloromethane, extracted once with saturated $NaHCO_3$ and saturated NaCl and dried over $MgSO_4$. The solvent is removed *in vacuo*. The product is purified on a silica gel column or by HPLC. The product is analyzed by ^1H, ^{13}C, and ^{13}C-APT NMR and mass spectroscopy.

25

EXAMPLE 54

Attachment of Thiol Linker at Position 2 and 8 of Purines

a.   2-Fluorohypoxanthene is treated with ethyl bromoacetate as per the procedures of Example **18** to give 9-carboxymethyl-2-fluorohypoxanthene ethyl ester

30   which is further reacted with N-(N-Boc-2-aminoethyl) glycine ethyl ester as per the

procedures of Examples **20** and **21** to give N-(N-Boc-2-aminoethyl)-N-[(2-fluorohypo-

xanthene-9-yl)acetyl]glycine. N-(N-Boc-2-aminoethyl)-N-[(2-fluorohypoxanthene)-

acetyl]glycine is reacted with 6-S-tritylthiohexylamine following the procedures of

Harris, *et al.*, *J. Am. Chem. Soc.* **1991**, *113*, 4328. The resulting purine C-2 thiol linker,

5   PNA monomer is incorporated into PNA oligomers.

        b.   8-Bromoadenine is treated with ethyl bromoacetate as per the procedures

of Example **18** to give 9-carboxymethyl-8-bromoadenine ethyl ester. . 9-Carboxymethyl-

8-bromoadenine ethyl ester is treated as per the procedures of Example **19** to put a Cbz

protection group on the N-6 exocyclic amino group. The resulting N-6-Cbz-9-

10   carboxymethyl adenine ethyl ester is further reacted with N-(N-Boc-2-aminoethyl)

glycine ethyl ester as per the procedures of Examples **20** and **21** to give N-(N-Boc-2-

aminoethyl)-N-[({N-6-Cbz-8-bromo}-9-adenyl)acetyl]glycine ethyl ester. N-(N-Boc-2-

aminoethyl)-N-[({N-6-Cbz-8-bromo}-9-adenyl)acetyl]glycine ethyl ester is further

reacted with 6-S-tritylthiohexylamine following the procedures of Harris, *et al.*, *J. Am.*

15   *Chem. Soc.* **1991**, *113*, 4328. The resulting purine C-8 thiol linker, PNA monomer is

incorporated into PNA oligomers.

EXAMPLE 55

Attachment Of Amino Linker At 6-Position of Purines

20   **b. N-(N-Boc-2-aminoethyl)-N-[((N-2-trifluoroacetyl)N-6-trifluoroacetyl-**
        ⁄
aminohexylamino-9-guaninyl)acetyl]glycine (55A)

        Guanine is dissolved in pyridine and treated with trifluoroacetic anhydride. The

resultant 2(trifluoroacetamido)--6-O-pyrilyl-guanine intermediate is further reacted with

pentafluorophenol to give 6-pentafluorophenyloxy-2-trifluoroacetamido guanine. A

25   similar reaction scheme has been previously described for 2'-deoxy guanosine by Gao,

et.al., *J. Org. Chem.*, **1992**, *57*, 6954.

        The 2-(protected)functionalized 6-protected nucleobase is further reacted with 2-

bromoethylacetate as per the procedures of Example **18** to give a protected acetyl tether

at the 9 position. The compound is treated with N-6-trifluoroacetyl-hexane-1,6-diamine

30   resulting in the replacement of the O-C_6F_5 with a bifunctional linking group

(NH(CH$_2$)$_6$NHCOCF$_3$). This monoprotected diamine linker has been described by Agrawal, *et.al.*, *Tetrahedron Lett.*, **1990**, *31*, 1543. After the protected linking group is attached the resulting compound is treated via the procedures of Example 22 to give the title compound. The final compound having a protected linking moiety is further

5   incorporated into oligomers using standard methods. The protected linking moiety is deprotected and functionalized with a further functional group e.g. fluorescein or biotin.

b. N-(N-Boc-2-aminoethyl)-N-[(N-6-trifluoroacetylaminohexylamino-9-adeninyl)acetyl]glycine (55B)

Hypoxanthine (6-hydroxypurine) is dissolved in pyridine and treated with

10   pentafluorophenol to give 6-pentafluorophenyloxy-purine. The modified nucleobase is further treated with 2-bromoethylacetate as per the procedures of Example **18** to give the protected acetyl tether at the 9 position. The compound is treated with N-6-trifluoroacetyl-hexane-1,6-diamine resulting in the replacement of the O-C$_6$F$_5$ with a bifunctional linking group (NH(CH$_2$)$_6$NHCOCF$_3$). After the protected linking group is

15   attached the resultant functionalized adenine nucleobase is treated via the procedures of Example 22 to give the title compound. The final compound having a protected linking moiety is further incorporated into oligomers using standard methods. The protected . linking moiety is deprotected and functionalized with a further functional group e.g. fluorescein or biotin.

20

EXAMPLE 56

Coupling of Cholesterol to Thiol Containing PNA Monomer via Disulfide Bridge

Compound **53A** or **53B** is treated with 2,2'-dithiobis(5-nitropyridine) in methylene chloride overnight. The precipitated 5-nitro pyridine-2-thione is removed by

25   filtration, and the filtrate is concentrated. The resultant product (**56C** or **56D**, respectively) is treated with thiocholesterol in CH$_2$Cl$_2$ and shaken overnight to give the compound (**56A** or **56B**, respectively) with cholesterol attached through a disulfide link. The resulting compounds are further used in PNA synthesis.

30   **EXAMPLE 57**

Conversion Of The Thiol Linker With A Trityl Protecting Group Into A Thiol

Linker Protected by Disulfide Linkage

Compound **56C** or **56D** is treated with propylmercaptan in CH_2Cl_2 and stirred

overnight. The resulting disulfide compound (**57A** and **57B**, respectively), which has a

5      $CH_2\text{-}O\text{-}(CH_2)_6\text{-}S\text{-}S\text{-}CH_2\text{-}CH_2\text{-}CH_3$ linkage, is further derivatized and incorporated into

PNA oligomers. The free thiol group is liberated, before conjugation, by the addition of

dithiothreitol (DTT).

EXAMPLE 58

10   **Conversion To A Base Labile Thiol linker**

Compound **56C** or **56D** is treated with CBzCl (carbobenzyloxy chloride) in

triethylamine. The resulting compound **58A** or **58B**, which contains a protected thiol

group is used in the synthesis of PNA oligomers. The free thiol group may be

regenerated from the monomer before or after the monomer is used for incorporation

15   into a PNA oligomer by treatment with ammonia followed by DTT.

EXAMPLE 59

Conjugation Reactions Of PNA-O-hexylthiol Linker

To illustrate the conjugation potential of the PNA-*O*- thiol tether, PNA H_2N-

20   GCA*T-COOH (SEQ ID NO:113) is synthesized as per the procedures of Example **26**.

The astrisk (*) denotes the incorporation of a disulfide protected thiolhexyloxymethyl

linker attached at the C-1 position of the 2-aminoethyl portion of the monomer in the

oligomer, synthesized as per the procedures of Example **58**. The PNA is treated with

$0.1M$ $AgNO_3$ in TEAA buffer followed by DTT treatment to generate a free thiol group.

25   The thiol-PNA is then reacted with four classes of compounds each having a

haloacetamide or a maleimide group and the desired functionality at the other end. The

following compounds are employed: (1) a phospholipid maleimide, which can offer cell

signaling and trafficking properties to PNAs; (2) 5-iodoacetamido-*O*-phenanthroline,

which is a nucleic acid cleaving agent; this particular conjugation offers an added

30   advantage of optimal placement for the cleaving agent as this reagent when complexed

to cuprous ion reacts via a minor groove attack at the C-1'position; (3) pyrene-

maleimide, which may stabilize a PNA-PNA, PNA-DNA or PNA-RNA duplex via

intercalation; and (4) fluorescein maleimide, which is used as a general diagnostic tool,

allowing the monitoring of PNA uptake. The conjugations are carried out in phosphate

5   buffer (pH 8.0). Completion of reaction is monitored by HPLC analysis using a C-18

column and a linear 1% increase of CH_3CN concentration for every minute. The

conjugates are purified by size exclusion and reverse phase HPLC and characterized by

their UV-VIS spectra (where applicable). Fluorescein maleimide, pyrene maleimide and

phospholipid maleimide are purchased from Molecular Probes (Eugene, Oregon). *O*-

10   Phenanthroline-5-iodoacetamide is synthesized according to the published procedure of

Sigman, *Biochemistry* **1990**, *29*, 9097.

EXAMPLE 60

N-[(N-Boc)-1-(5-phthaloylpentyloxymethyl)-2-aminoethyl]-N-[(1-

15   **thyminyl)acetyl]glycine Ethyl ester (57) and N-[(N-Boc)-2-(5-**

phthaloylpentyloxymethyl)-2-aminoethyl]-N-[(1-thyminyl)acetyl]glycine Ethyl ester

(57A)

        Compounds **35** and **36** are independently treated with ethanol/DCC to produce

the ethyl ester which is then treated with 5-bromopentylphthalimide to yield the title

20   compounds.

EXAMPLE 61

N-[(N-Boc)-1-(5-phthaloylpentyloxymethyl)-2-aminoethyl]-N-[(9-adeninyl)-

acetyl]glycine Ethyl ester (61) and N-[(N-Boc)-2-(5-phthaloylpentyloxymethyl)-2-

25   **aminoethyl]-N-[(9-adeninyl)-acetyl]glycine Ethyl ester (61A)**

        Compounds **61** and **61A**, are prepared using the general procedures of Examples

1 thru **6**, **35** and **36**, and further treated according to the procedures of Example **60**, to

yield the intermediate ethyl esters and the title compounds.

30   **EXAMPLE 62**

N-[(N-Boc)-1-(5-aminopentyloxymethyl)-2-aminoethyl]-N-[(9-adeninyl)-acetyl]glycine Ethyl ester (62) and N-[(N-Boc)2-(5-aminopentyloxymethyl)-2-aminoethyl]-N-[(9-adeninyl)-acetyl]glycine Ethyl ester (62A)

Compound **61** or **61A** is dissolved in 200 ml methanol in a 500 ml flask.

5   Hydrazine is added to the stirring reaction mixture. The mixture is heated to 60-65° in an oil bath and refluxed 14 hours. Solvent is evaporated *in vacuo*. The residue is dissolved in dichloromethane (250 ml) and extracted twice with an equal volume NH_4OH. The organic layer is evaporated to yield the crude product. NMR is used to assay product purity. The products are used in subsequent reactions without further

10   purification.

EXAMPLE 63

N-[(N-Boc)-1-(5-phthaloylpentyloxymethyl)-2-aminoethyl]-N-[(1-thyminyl)-acetyl]glycine methyl ester (63)

15   Substrate **42** is converted to the title compound as per the procedures of Example **60.**

EXAMPLE 64

N-[(N-Boc)-1-(5-aminopentyloxymethyl)-2-aminoethyl]-N-[(1-thyminyl)-

20   **acetyl]glycine methyl ester (64)**

Substrate **63** is converted to the title compound as per the procedures of Example **62.**

EXAMPLE 65

25   **Synthesis of PNA Oligomer Having Amine Containing Functionalities**

The following PNA oligomers having aminoethylglycine backbones are synthesized according to the procedures of Examples **26** et seq.:

H_2N-GCA* TGC AT-COOH (SEQ ID NO:60),

H_2N-GCA T**GC AT-COOH (SEQ ID NO:61),

30   H_2N-GCA T***GC A***T-COOH (SEQ ID NO:62)

H$_2$N-CTG TCT CCA* TCC TCT TCA CT-COOH (SEQ ID NO:63)

H$_2$N-CTG TCT CCA*** TCC TCT TCA*** CT-COOH (SEQ ID NO:64)

H$_2$N-CCA A**GC CUC AGA-COOH (SEQ ID NO:65)

H$_2$N-CCA GGC UCA GA*T-COOH (SEQ ID NO:66)

5   wherein a *, **, or *** represents a PNA monomer functionalized to incorporate a pentyl-N-phthalimido functionality at a position having a hydroxy methyl group. A * indicates a pentyl-N-phthalimido functionality attached to the hydroxy methyl group at the C-1 carbon of the 2-aminoethyl portion of the monomer as synthesized in Examples **60** and **61**. A ** indicates a pentyl-N-phthalimido functionality attached to the hydroxy methyl

10   group at the C-2 carbon of the 2-aminoethyl portion of the monomer as synthesized in Examples **60** and **61**. A *** indicates a pentyl-N-phthalimido functionality at serine-O of the serine portion of the monomer as synthesized in Example **63**. PNA Oligomers SEQ ID NO:63 and 64 are compounds complementary to the E2 region of the bovine papilloma virus-1 (BPV-1). PNA oligomers SEQ ID NO:65 and 66 are compounds

15   complementary to the HIV-1 TAR region.

EXAMPLE 66

Conjugation Of PNA Oligomers Through a Hydroxyl Group

A. Functionalization with Biotin

20   ### 1. Single Site Modification

About 10 O.D. units (A$_{260}$) of PNA oligomer SEQ ID NO:63 is treated as per the procedures of Example **62** to remove the phthaloyl protecting group. The resulting oligomer (*see*, Example **65**) is dried in a microfuge tube. The PNA oligomer is dissolved in 200 µl of 0.2 M NaHCO$_3$ buffer and D-biotin-N-hydroxysuccinimide ester

25   (2.5 mg, 7.3 µmols) (Sigma, St. Louis, MO) is added followed by 40 µl DMF. The solution is allowed to stand overnight. The solution is then applied to a Sephadex G-25 column (0.7 x 15 cm) and the PNA oligomer fractions are combined. Analytical HPLC is used to determine the percentage conversion to the product. The product is purified by HPLC (Waters 600E with 991 detector, Hamilton PRP-1 column 0.7 x 15 cm;

30   solvent A: 50 mM TEAA pH 7.0; B: 45 mM TEAA with 80% acetonitrile: 1.5 ml flow

rate: Gradient: 5% B for first 5 minutes, linear (1%) increase in B every minute

thereafter) and further desalted on Sephadex G-25 to give the PNA oligomer:

CTG TCT CCA* TCC TCT TCA CT (SEQ ID NO:67)

wherein A* represents a PNA monomer functionalized to incorporate a biotin

5   functionality linked via a pentylamino linking group attached to the C-1 hydroxymethyl

group of the PNA monomer.

2. Multiple Site Modification

About 10 O.D. units (A$_{260}$) of PNA oligomer SEQ ID NO:64 is treated as per the

procedures of Example **62** to remove the phthaloyl protecting group. The resulting

10   oligomer (*see*, Example **65**) is treated utilizing the method of Example **61(A)(1)** above

with D-biotin-N-hydroxysuccinimide ester (5 mg) in 300 μl of 0.2 M NaHCO$_3$ buffer/

50 μl DMF. Analytical HPLC is used to monitor progress of the reaction. HPLC and

Sephadex G-25 purification give the PNA oligomer:

CTG TCT CCA* TCC TCT TCA* CT (SEQ ID NO:68)

15   wherein A* represents a PNA monomer functionalized to incorporate a biotin

functionality linked via a pentylamino linking group to the hydroxyl group of the serine

portion of the PNA.

B. Functionalization with Fluorescein

1. Single Site Modification

20   A 1M Na$_2$CO$_3$/1M NaHCO$_3$ buffer (pH 9.0) is prepared by adding 1M NaHCO$_3$

to 1M Na$_2$CO$_3$. A 200 μl portion of this buffer is added to 10 O.D. units of PNA

oligomer SEQ ID NO:63 (which is first treated to remove the phthaloyl protecting group

as above) (*see*, Example **65**) in a microfuge tube. A 10 mg portion of fluorescein-

isothiocyanate in 500 μl DMF is added to give a 0.05 M solution. A 100 μl portion of

25   the fluorescein solution is added to the solution of PNA oligomer in the microfuge tube.

The tube is covered with aluminum foil and allowed to stand overnight. The reaction

mixture is applied to a Sephadex G-25 column (0.7 x 20 cm) that has been equilibrated

with 25% (v/v) ethyl alcohol in water. The column is eluted with the same solvent.

Product migration can be seen as a yellow band well separated from dark yellow band of

30   the excess fluorescein reagent. The fractions showing absorption at 260 nm and 485 nm

are combined and purified by HPLC as per the purification procedure of Example

66(A)(1). The product is lyophilized and desalted on Sephadex to give the PNA

oligomer:

CTG TCT CCA* TCC TCT TCA CT (SEQ ID NO:69)

5   wherein A* represents a PNA monomer functionalized to incorporate a fluorescein

functionality linked via a pentylamino linking group to the hydroxymethyl group at the

C-1 position of the 2-aminoethyl portion of the PNA.

2. Multiple Site Modification

About 10 O.D. units (A_{260}) of PNA oligomer SEQ ID NO:64 is treated as per the

10   procedures of Example **62** to remove the phthaloyl protecting group. The resulting

oligomer (see Example **60**) is dissolved in 300 µl of the 1M Na_2HCO_3/ 1M Na_2CO_2

buffer of Example **61(B)(1)** and 200 µl of the fluorescein-isothiocyanate stock solution

of Example **61(B)(1)** is added. The resulting solution is treated as per Example

61(B)(1). Analytical HPLC is used to indicate the percent of doubly labeled product.

15   Work up of the reaction gives the PNA oligomer:

CTG TCT CCA* TCC TCT TCA* CT (SEQ ID NO:70)

wherein A* represents a PNA monomer functionalized to incorporate a fluorescein

functionality linked via an pentylamino linking group to the hydroxyl group of the serine

portion of the PNA.

20   **C. Functionalization with Cholic Acid**

1. Cholic Acid N-Hydroxysuccinimide Ester (Compound 61(C)(1)(A))

Anhydrous DMF (150ml) was added to a mixture of cholic acid (4.09g, 15mmol)

and N-hydroxysuccinimide (5.25g, 45 mmol). The mixture was stirred in the presence

of nitrogen. EDAC (4ml, 25mmol) was then added and the mixture was stirred

25   overnight. The solution was then evaporated to a gum and partitioned between 1:1 ethyl

acetate and 4% $NaHCO_3$ solution (pH 7.9) (100ml each). The organic layer was washed

with saturated NaCl solution, dried over anhydrous Na_2SO_4 and evaporated to yield the

title compound as a pale yellow foam (4.6g, 91%). ^{13}C NMR (DMSO-d_6) δ 12.27, 16.71,

22.58, 22.80, 25.42, 26.19, 27.20, 28.49, 30.41, 30.56, 34.36, 34.82, 34.82, 35.31, 39.09,

30   39.09, 41.38, 41.53, 45.84, 46.05, 66.25, 70.45, 71.03, 169.28 and 170.16.

2. Single Site Modification

About 10 O.D. units (A_{260}) of PNA oligomer SEQ ID NO:63 is treated as per the procedures of Example **62** to remove the phthaloyl protecting group. The resulting oligomer (*see*, Example **60**) is treated with cholic acid-NHS ester (Compound **1** in Application Serial Number 782,374, 5 mg, 9.9 μmols) in 200 μl of 0.2 M NaHCO$_3$ buffer/40 μl DMF. The reaction mixture is heated for 16 hours at 45°C. Analytical HPLC is used to determine the percent of product formation. Work up of the reaction gives the PNA oligomer:

CTG TCT CCA* TCC TCT TCA CT (SEQ ID NO:71)

wherein A* represents a PNA monomer functionalized to incorporate a cholic acid functionality linked via a pentylamino linking group to the hydroxymethyl group at the C-1 position of the 2-aminoethyl portion of the PNA.

3. Multiple Site Modification

About 10 O.D. units (A_{260}) of PNA oligomer SEQ ID NO:64 is treated as per the procedures of Example **62** to remove the phthaloyl protecting group. The resulting oligomer (*see*, Example **65**) is treated with cholic acid-NHS ester (Compound **1** in Application Serial Number 782,374, 10 mg, 19.8 μmols) in 300 μl of 0.2 M NaHCO$_3$ buffer/ 50 μl DMF. The reaction mixture is heated for 16 hours at 45°C. The product is isolated according to the method of Example **66(A)(1)**. Analytical HPLC is used to determine the percentages of doubly and singly labeled products. Work up according to the procedure of Example **66(A)(1)** gives the PNA oligomer:

CTG TCT CCA* TCC TCT TCA* CT (SEQ ID NO:72)

wherein A* represents a PNA monomer functionalized to incorporate a cholic acid functionality linked via a pentylamino linking group to the hydroxyl group of the serine portion of the PNA.

D. Functionalization with Digoxigenin

1. Single Site Modification

About 10 O.D. units (A_{260}) of PNA oligomer SEQ ID NO:63 is treated as per the procedures of Example **62** to remove the phthaloyl protecting group. The resulting oligomer (*see*, Example **65**) is treated with digoxigenin-3-O-methylcarbonyl-ε-

aminocaproic N-hydroxy succinimide ester (Boehringer Mannheim Corporation,

Indianapolis, IN) in 200 μl of 0.1 M borate pH 8.3 buffer/ 40 μl DMF. The reaction

mixture is allowed to stand overnight. The product is isolated according to the method

of Example **66(A)(1)**. Work up of the reaction gives the PNA oligomer:

5   CTG TCT CCA* TCC TCT TCA CT (SEQ ID NO:73)

wherein A* represents a PNA monomer functionalized to incorporate a digoxigenin

functionality linked via an pentylamino linking group to the hydroxymethyl group at the

C-1 position of the 2-aminoethyl portion of the PNA.

2. Multiple Site Modification

10   About 10 O.D. units (A_{260}) of PNA oligomer SEQ ID NO:64 is treated as per the

procedures of Example **62** to remove the phthaloyl protecting group. The resulting

oligomer (*see*, Example **30**) is treated with digoxigenin-3-O-methylcarbonyl-ε-

aminocaproic N-hydroxy succinimide ester (Boehringer Mannheim Corporation,

Indianapolis, IN) in 300 μl of 0.1 M borate pH 8.3 buffer/50 μl DMF. The reaction

15   mixture was allowed to stand overnight. The product was isolated according to the

method of Example **66(A)(1)**. Work up as per Example **66(A)(1)** gives the PNA

oligomer:

CTG TCT CCA* TCC TCT TCA* CT (SEQ ID NO:74)

wherein A* represents nucleotides functionalized to incorporate a cholic acid

20   functionality linked via a pentylamino linking group to the hydroxyl group of the serine

portion of the PNA.

EXAMPLE 67

Functionalization of PNA Monomers with Pyrene

25   **N-[(N-Boc)-1-{(5-{4-(1-pyrenyl)butanoylamino}pent-1-yl)oxymethyl}-2-**
aminoethyl]-N-[(uracil-1-yl)acetyl]glycine ethyl ester (67) and N-[(N-Boc)-2-{(5-{4-
(1-pyrenyl)butanoylamino}pent-1-yl)oxymethyl}-2-aminoethyl]-N-[(uracil-1-
yl)acetyl]glycine ethyl ester (67A)

         A. Preparation of N-[(N-Boc)-1-(5-aminopentyloxymethyl)-2-aminoethyl]-

30   **N-[(uracil-1-yl)acetyl]glycine ethyl ester (67a) and N-[(N-Boc)-2-(5-aminopentyl-**

oxymethyl)-2-aminoethyl]-N-[(uracil-1-yl)acetyl]glycine ethyl ester (67b)

The title compounds are synthesized as per the procedures of Examples 1 thru 11, 35, 39, 60, and 62.

B.   Preparation of N-[(N-Boc)-1-{(5-{4-(1-pyrenyl)butanoylamino}pent-1-

5   **yl)oxymethyl}-2-aminoethyl]-N-[(uracil-1-yl)acetyl]glycine ethyl ester (67)**

Compound **67a** or **67b** (0.78 mmol), are dissolved in anhydrous DMF (15 mL). 1-Hydroxybenzotriazole (1.17 mmol) and 1-pyrene-butyric acid pentafluorophenyl ester (1.17 mmol) are added to the reaction mixture. The mixture is stirred under argon at room temperature for 2 hours, after which it is concentrated *in vacuo*. Residual DMF is

10   coevaporated with toluene. The residue is dissolved in dichloromethane (50 mL) and washed with an equal volume saturated $NaHCO_3$. The aqueous layer is washed with dichloromethane and the combined organic extracts are washed with an equal volume saturated NaCl. The aqueous layer is washed with dichloromethane and the combined organic layers dried over $MgSO_4$ and concentrated. The residue is purified by silica gel

15   column chromatography to yield the respective product.

EXAMPLE 68

A.   Preparation of N-[[(N-Boc)-1-[(5-(5-fluorosceinylthioureido)-pent-1-yl)oxymethyl]-2-aminoethyl]-N-[(uracil-1-yl)acetyl]glycine ethyl ester (68)

20   Fluorescein isothiocyanate (Isomer I, available from Cal Biochem, La Jolla, CA) was treated with 12 equivalents of pivaloyl chloride in Et_3N/THF to give di-O-pivaloyl fluorescein isothiocyanate. This compound was purified using a silica gel column with 3:1 hexane:ethyl acetate. Compound **67a** is then condensed with dipivaloyl fluorescein isothiocyanate in CH_2Cl_2/pyrimidine. The protecting groups are removed by treatment

25   with dilute acid. The title compound is then purified by elution from a silica column using ethyl acetate hexane as the eluent.

B.   Preparation of N-[[(N-Boc)-2-[(5-(5-fluorosceinylthioureido)-pent-1-yl)oxymethyl]-2-aminoethyl]-N-[(uracil-1-yl)acetyl]glycine ethyl ester (68A)

Substituting **67b** for **67a** into the above procedure will give the title compound.

EXAMPLE 69

A.   **Preparation of N-[(N-Boc)-1-[1-({N-(cholesterol-O-carboxy)-5--aminopentyl}oxymethyl)-2-aminoethyl]-N-[(uracil-1-yl)acetyl]]glycine ethyl ester (69)**

5   Compound **67a** (6.0 mmol) is dissolved in anhydrous pyridine/dichloromethane 50/50 (v/v) (20 mL). Cholesteryl chloroformate (Fluka, 6.68 mmol) is dissolved in anhydrous dichloromehthane (20 ml) and added slowly under argon with a syringe to the stirring reaction mixture. The mixture is stirred under argon at room temperature for 2 h after which it is concentrated *in vacuo*. Residual DMF is coevaporated with toluene.

10   The residue is dissolved in dichloromethane (50 mL) and washed with an equal volume of saturated $NaHCO_3$. The aqueous layer is washed with dichloromethane and the combined organic extracts washed with an equal volume of saturated NaCl. The aqueous layer is washed with dichloromethane and the combined organic layers dried over $MgSO_4$ and concentrated. The residue is chromatographed on a silica gel column

15   with a gradient of 25% ethyl acetate in hexanes to 100% ethyl acetate. The desired product elutes with 100% ethyl acetate and is assayed for purity by TLC.

B.   **Preparation of N-[(N-Boc)-2-[1-({N-(cholesterol-O-carboxy)-5--aminopentyl}oxymethyl)-2-aminoethyl]-N-[(uracil-1-yl)acetyl]]glycine ethyl ester (69a)**

20   Substituting **67b** for **67a** into the above procedure will give the title compound.

EXAMPLE 70

A.   **Preparation of N-[(N-Boc)-1-[{N-(2,4-dinitrophenyl)-5-aminopentyl}-oxymethyl]-2-aminoethyl]-N-[(uracil-1-yl)acetyl]glycine ethyl ester (70)**

25   Compound **67a** (1.37 mmol) is dissolved in methanol (20 mL). 2,4-Dinitrofluorobenzene (DNFB, 0.25 grams, 1.37 mmol) is added and the mixture shaken on a mechanical shaker. The reaction is monitored by TLC. After 90 min, another 0.25 grams of DNFB is added and the reaction mixture shaken an additional 30 min, followed by addition of another 0.25 grams of DNFB. After shaking 2.5 hours, the

30   mixture is concentrated *in vacuo* and chromatographed on a silica gel column, eluting

with a gradient of 25% ethyl acetate in hexanes to 100% ethyl acetate. The desired product elutes with 100% ethyl acetate and is assayed for purity by TLC.

B.   Preparation of N-[(N-Boc)-2-[{N-(2,4-dinitrophenyl)-5-aminopentyl}-oxymethyl]-2-aminoethyl]-N-[(uracil-1-yl)acetyl]glycine ethyl ester (70A)

5   Substituting **67b** for **67a** into the above procedure will give the title compound.

EXAMPLE 71

A.   Preparation of N-[(N-Boc)-1-[(N-(N_α-N_{im}-Di-FMOC-$_L$-histidinyl)-5-aminopentyl)oxymethyl]-2-aminoethyl]-N-[(uracil-1-yl)acetyl]glycine ethyl ester

10   **(71)**   Compound **67a** (1.51 mmol) is dissolved in dichloromethane (25 mL) and cooled to 0°C in an ice bath. Nα, Nim-Di-FMOC-$_L$-histidine pentafluorophenyl ester (3.1 mmol, purchased from Sigma) and 1-hydroxybenzotriazole (0.24 mmol, purchased from Fluka) are added to the stirred reaction mixture stirred under argon. After 15 minutes, the ice bath is removed and the mixture stirred under argon at room

15   temperature for 72 h. The mixture is concentrated *in vacuo* and chromatographed on a silica gel column, eluting with a gradient of 50% ethyl acetate in hexanes to 70% ethyl acetate in hexanes. The desired product elutes with 70% ethyl acetate.

B.   Preparation of N-[(N-Boc)-2-[(N-(N_α-N_{im}-Di-FMOC-$_L$-histidinyl)-5-aminopentyl)oxymethyl]-2-aminoethyl]-N-[(uracil-1-yl)acetyl]glycine ethyl ester

20   **(71A)**

Substituting **67b** for **78a** into the above procedure will give the title compound.

EXAMPLE 72

A.   Preparation of N-[[(N-Boc)-1-[(N-(Ω-methyl-polyethylene glycol-

25   **propionoyl)-5-aminopentyl)oxymethyl]]-2-aminoethyl]-N-[(uracil-1-yl)acetyl]glycine ethyl ester (72)**

Compound 67a, (1.55 mmol) is dissolved in anhydrous DMF (15 mL). 1-Hydroxybenzotriazole (1.75 mmol) and polyethylene glycol-propionic acid-NHS-ester (1.75 mmol) are added to the reaction mixture. The mixture is stirred under argon at

30   room temperature for 2 hours after which it is concentrated *in vacuo*. Residual DMF is

coevaporated with toluene. The residue is dissolved in dichloromethane (50 mL) and then washed with an equal volume saturated NaHCO₃. The aqueous layer is washed with dichloromethane and the combined organic extracts washed with an equal volume saturated NaCl. The aqueous layer is washed with dichloromethane and the combined

5   organic layers dried over $MgSO_4$ and concentrated. The residue is chromatographed on a silica gel column, eluting with a gradient of 50% ethyl acetate in hexanes to 100% ethyl acetate.

      B.   **Preparation of N-[[(N-Boc)-2-[(N-(Ω-methyl-polyethylene glycol-propionoyl)-5-aminopentyl)oxymethyl]]-2-aminoethyl]-N-[(uracil-1-**

10   **yl)acetyl]glycine ethyl ester (72a)**

      Substituting **67b** for **67a** into the above procedure will give the title compound.

EXAMPLE 73

      A.   **Preparation of Macrocycle derivatized PNA monomer**

15   Compound **67a**, (1.55 mmol) is treated according to the procedures of Example **67** with the macrocycle 4-{1,4,8,11-tetraza-[tri-(trifluoroacetyl)cyclotetradec-1-yl]}methyl benzoic acid-N-hydroxy succinimide ester (prepared according to Simon Jones, *et. al.*, *Bioconjugate Chem.* **1991**, *2*, 416) to yield the product.

      Substituting **67b** for **67a** into the above procedures will give the 2 substituted

20   compound.

EXAMPLE 74

Functionalization with Folic Acid

      A.   **Preparation of N-[(N-Boc)-1-[(5-(N-folate)aminopentyl)oxymethyl]-**

25   **2-aminoethyl]-N-[(uracil-1-yl)acetyl]glycine ethyl ester (74a)**

      Compound **67a**, is treated according to the procedures of Example **67** with folic acid pentafluorophenyl ester (protected with an isobutyryl protecting group) to yield the product.

      B.   **Preparation of N-[(N-Boc)-2-[(5-(N-folate)aminopentyl)oxymethyl]-**

30   **2-aminoethyl]-N-[(uracil-1-yl)acetyl]glycine ethyl ester (74b)**

Substituting **67b** for **67a** into the above procedures will give 2 substituted compound.

EXAMPLE 75

5   **A.   N-[(N-Boc)-1-[(N-(2-methoxy-6-chloro-9-(6-aminohexanoyl)acridine)-5-aminopentyl)oxymethyl]-2-aminoethyl]-N-[(uracil-1-yl)acetyl]glycine ethyl ester (75)**

6,9-Dichloro-2-methoxyacridine (Aldrich, 36 mmol) and phenol (2.5 g) were placed together on a round-bottom flask with a stirring bar and to this 6-amino-hexanoic

10   acid (9.3 g, 71 mmol) was added and the flask was heated to 100° (oil bath) for 2 hours. TLC (10% methanol in methylene chloride) showed complete disappearance of starting material. The reaction mixture was cooled and poured into 200 mL of methanol. The product isolates out as a yellow solid (about 10 g). This compound was then converted into its pentafluorophenol ester.

15   Compound **67a** (78 mmol) is dissolved in anhydrous DMF (15 mL). 1-Hydroxy-benzotriazole (1.17 mmol) and 2-methoxy-6-chloro-9-(6-aminohexanoyl)acridine pentafluorophenyl ester (1.17 mmol) are added to the reaction mixture. The mixture is stirred under argon at room temperature for 2 h, after which it is concentrated *in vacuo*. Residual DMF is coevaporated with toluene. The residue is dissolved in

20   dichloromethane (50 mL) and washed with an equal volume of saturated $NaHCO_3$. The aqueous layer is washed with dichloromethane and the combined organic extracts washed with an equal volume of saturated NaCl. The aqueous layer is then washed with dichloromethane and the combined organic layers dried over $MgSO_4$ and concentrated. The residue is purified by silica gel column chromatography.

25   **B.   Preparation of N-[(N-Boc)-2-[(N-(2-methoxy-6-chloro-9-(6-aminohexanoyl)acridine)-5-aminopentyl)oxymethyl]-2-aminoethyl]-N-[(uracil-1-yl)acetyl]glycine ethyl ester (75a)**

Substituting **62b** for **62a** into the above procedures will give 2 substituted compound.

30

EXAMPLE 76

A.   Preparation of N-[(N-Boc)-1-{(-5-(N,N-dimethylamino)pentyl)-oxymethyl}-2-aminoethyl]-N-[(uracil-1-yl)acetyl]glycine ethyl ester (76)

Compound **67a** (0.29 mmol) is dissolved in 4 ml methanol. Sodium acetate pH

5   4.0 (2 ml), sodium cyanoborohydride (0.3 mmol) and 37% formaldehyde in water (300

µl) are added to the reaction mixture, which is stirred 2 hours, after which it is

concentrated *in vacuo*. The residue is dissolved in dichloromethane (50 mL) and

washed with an equal volume saturated $NaHCO_3$. The aqueous layer is washed with

dichloromethane and the combined organic extracts washed with an equal volume

10   saturated NaCl. The aqueous layer is washed with dichloromethane and the combined

organic layers dried over $MgSO_4$ and concentrated. The residue is chromatographed on

a silica gel column, eluting with a gradient of 50% ethyl acetate in hexanes to 100%

ethyl acetate. The desired product elutes with 10% Methanol-90% ethyl acetate.

A.   Preparation of N-[(N-Boc)-2-{(-5-(N,N-dimethylamino)pentyl)-

15   oxymethyl}-2-aminoethyl]-N-[(uracil-1-yl)acetyl]glycine ethyl ester (76a)

Substituting **67b** for **67a** into the above procedures will give 2 substituted

compound.

EXAMPLE 77

20   **Functionalization of PNA Oligomer with Reporter Enzymes, Peptides and Proteins**

A. Use of Heterobifunctional Linker

1. Synthesis of PNA-Maleimide Conjugate

PNA oligomer SEQ ID NO:63 is treated as per the procedures of Example **62** to remove the phthaloyl protecting group. The resulting oligomer is lyophilized in a 5 ml pear-shaped flask. Sulfo-SMCC reagent, Pierce Chemical Co. (Rockford, Il) (46 µmols) is dissolved in phosphate buffer (800 µl, 0.1M, pH 7.0) and added to the PNA oligomer

5   bearing flask. An additional 200 µl of buffer are used to wash the reagent and transfer it to the flask containing the PNA oligomer. The contents of the flask are stirred overnight and loaded on to a Sephadex G-25 column (1 x 40 cm) equipped with a fraction collector. The PNA oligomer-maleimide conjugate containing fractions are collected and tested by analytical HPLC for separation from other NHS type products.

10   **2. Synthesis of PNA oligomer-Peptide Conjugate**

An aliquot of the PNA oligomer-maleimide conjugate of Example **77(A)(1)** (300 nmols) is lyophilized in a microfuge tube. SV40 peptide (pro-asp-lys-lys-arg-lys-cys)(about 2.5 µmols) is taken up in phosphate buffer (800 µl, 0.1 M, pH 7.0) and added to the PNA oligomer-maleimide conjugate containing tube. The contents of the tube are

15   stirred overnight under an argon atmosphere. The reaction mixture is passed through a Sephadex G-25 column and the PNA-peptide conjugate fractions are identified by HPLC. Isolation of the product from product-bearing fractions via HPLC and desalting on Sephadex G-25 will yield a PNA oligomer of the sequence:

CTG TCT CCA* TCC TCT TCA CT SEQ ID NO:75

20   wherein A* represents a PNA monomer functionalized to incorporate a SV40 peptide functionality linked via a pentyl-amino-sulfo-SMCC (sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate) linking group to the hydroxymethyl group of the designated PNA monomer.

B. Use of Homobifunctional Linker

25   **1. Synthesis of PNA Oligomer-Disuccinimidyl Suberate Conjugate**

PNA oligomer SEQ ID NO:63 is treated as per the procedures of Example **62** to remove the phthaloyl protecting group. The resulting oligomer is evaporated to dryness and dissolved in freshly prepared 0.1 M $NaHCO_3$/50 mM EDTA (100 µl, pH 8.25). The solution is then treated with a solution of DSS, Pierce Chemical Co. (Rockford, Il) (7

5   µmol) in 200 µl DMSO. The solution is stored at room temperature for 15 minutes and then immediately applied to a Sephadex G-25 column (1 x 40 cm) which has been previously packed and washed with water at 4°C. The PNA oligomer fractions are combined immediately in a 25 ml pear-shaped flask and are rapidly frozen in dry ice/isopropyl alcohol and lyophilized to a powder.

10   **2. Synthesis of PNA Oligomer-Protein Conjugate**

A solution of calf intestinal alkaline phosphatase (Boehringer Mannheim) (2.06 ml, 147 nmol) is spun at 4°C in a Centricon microconcentrator at 6000 rpm until the volume is less than 50 µl. It is then redissolved in 1 ml of cold Tris buffer (pH 8.5, 0.1M containing 0.1 NaCl and 0.05 M $MgCl_2$) and concentrated twice more. Finally the

15   concentrate is dissolved in 400 µl of the same buffer. This solution is added to the activated PNA oligomer from Example **77(B)(1)** and the solution stored for 18 hrs at room temperature. The product is diluted to approximately 30 ml and applied to a Sephadex G-25 column (1 x 20 cm, chloride form) maintained at 4°C. The column is eluted with 50 nM Tris-Cl pH 8.5 until the UV absorbance of the fractions eluted, reach

20   near zero values. The column is then eluted with a NaCl salt gradient 0.05 M to 0.75 M (150 ml each). The different peaks are assayed for both PNA oligomer presence and alkaline phosphatase activity and the product-bearing fractions are combined. Typically the first peak will be excess enzyme, the second peak the PNA oligomer-protein conjugate and the third peak unreacted PNA oligomer. Isolation of the product from the

25   product-bearing fractions via HPLC and desalting on Sephadex G-25 will yield a PNA oligomer of the sequence:

CTG TCT CCA* TCC TCT TCA CT SEQ ID NO:76

wherein A* represents a PNA monomer functionalized to incorporate an alkaline phosphatase functionality linked via a DSS linking group to the aminopentyl oxymethyl

30   of the designated PNA monomer.

EXAMPLE 78

Retinoic Acid Conjugated PNA oligomers

A. Retinoic Acid N-Hydroxysuccinimide Ester

5   Anhydrous DMF (150 ml) was added to a mixture of retinoic acid (15 mmol,

Fluka) and N-hydroxysuccinimide (45 mmol). The mixture was stirred in the presence

of argon. EDAC [ethyl-3-(3-dimethylamino)propyl carbodiimide] (4 ml, 25 nmol) was

then added and this mixture was then stirred overnight. The solution was then

evaporated to a yellow gum and dissolved in 200 ml ethylacetate and washed

10   successively with 4% NaHCO$_3$ solution (200 ml) followed by saturated NaCl solution,

dried over anhydrous MgSO$_4$ and evaporated to yield the desired compound as a yellow

solid in nearly 90% yield.

B. Retinoic Acid Functionalized PNA Monomers

Compound **67a**, **67b**, **62**, or **62A** is dissolved in 200 μl of 0.2 M NaHCO$_3$ buffer

15   and retinoic acid N-hydroxysuccinimide ester (7.3 μmols) is added followed by 40 μl

DMF. The solution is allowed to stand overnight. The solution is then applied to a

Sephadex G-25 column (0.7 x 15 cm) and the PNA oligomer fractions are combined.

Analytical HPLC is used to determine the percentage conversion to the product. The

product is purified by HPLC (Waters 600E with 991 detector, Hamilton PRP-1 column

20   0.7 x 15 cm; solvent A: 50 mM TEAA pH 7.0; B: 45 mM TEAA with 80% acetonitrile:

1.5 ml flow rate: Gradient: 5% B for first 5 minutes, linear (1%) increase in B every

minute thereafter) and further desalted on Sephadex G-25 to give the retinoic acid-PNA

monomer conjugate.

C. Retinoic Acid Functionalized PNA Oligomers

25   The retinoic acid-PNA monomer conjugates of Example **78B** may be

incorporated into PNA oligomers by deprotection of the PNA monomer carboxyl group

and insertion of the resulting monomer into the synthetic procedures of Example **26**.

EXAMPLE 79

30   Folic Acid Conjugated Oligonucleotide

A mixture of folic acid (30 mg, 68 μmols) and 1-hydroxybenzotriazole (30 mg,

222 μmols) is dissolved in 9 ml of dry DMF. To this solution, 50 μL of EDAC (312

μmols) is added. The resultant yellow viscous material is vortexed well and 500 μL

from the solution is transferred into a 0.2M NaHCO$_3$ buffer solution containing PNA

5   oligomer SEQ ID NO:63 which has previously been treated according to the procedure

of Example **62** to convert the NPHTH group to the free amine. The solution is vortexed,

covered with aluminum foil and allowed to react for 16 hrs. The mixture is then loaded

onto a Sephadex G-25 column (1 x 40 cm). The PNA oligomer fraction is collected,

concentrated and passed one more time through a Sephadex G-25 column. Isolation of

10   the product from the product-bearing fractions via HPLC and desalting on Sephadex G-

25 will yield a PNA oligomer of the sequence:

CTG TCT CCA* TCC TCT TCA CT SEQ ID NO:77

wherein A* represents a PNA monomer functionalized to incorporate a folic acid

functionality linked to the aminopentyloxymethyl of the designated PNA monomer.

15

EXAMPLE 80

Methyl Folate Conjugated PNA Oligomer

In a like manner to Example **79**, 5-methyl folate is conjugated to deprotected

PNA oligomers SEQ ID NO:63 and SEQ ID NO:64 to give the conjugated methyl folate

20   group tethered to the C-1 position of the 2-aminoethyl portion of the PNA oligomer and

to the Serine O portion when SEQ ID NO:64 is used which also has multiple sites for

conjugation.

EXAMPLE 81

25   **Pyridoxal Conjugated PNA Oligomer**

PNA oligomer SEQ ID NO:63 or PNA oligomer SEQ ID NO:64 is treated

according to the procedure of Example **62** to convert the N-PHTH group to the free

amine. The resulting PNA oligomer is dissolved in 100 microliters of water, and 100 ml

of 1M NaOAc buffer (pH 5.0) is added followed by 5 mg of pyridoxal hydrochloride (24

30   μmols) and 50 μl of 60 mM NaCNBH$_3$ solution. The solution is vortexed, left

overnight, and is then passed through a Sephadex G-25 column and further purified in an analytical HPLC column.

EXAMPLE 82

Tocopherol Conjugated PNA Oligomer

A. Vitamin E (Tocopherol)-hemisuccinate-NHS ester

α-Tocopherolhemisuccinate (Sigma, 5 g, 9.4 mmols) was treated with 3 equivalents of N-hydroxysuccinimide and 2 equivalents of EDAC as described above in Example **78A**. The work-up is the same as for Example **51**, and yields the title compound as a light brown wax-like solid.

B. Tocopherol Conjugated PNA Oligomer

α-Tocopherol-hemisuccinate-NHS ester is treated according to the procedure of Example **78B** and **78C** above to obtain tocopherol attached via a tether to the desired PNA oligomer.

EXAMPLE 83

Conversion of a PNA Oligomer Having an Alkyl Aminolinker to a PNA Oligomer Having a Thiolinker

A. PNA Oligomer SEQ ID NO:78

PNA oligomer (SEQ ID NO:63) H_2N-CTG TCT CCA* TCC TCT TCA CT-COOH wherein * represents a pentyl N-phthaloyl oxymethyl group attached at the C-1 position of the 2-aminoethyl portion of the designated monomer in the oligomer, is treated with hydrazine/ethanol followed by 5 mg SATA (N-succinimidyl-S-acetylthio-acetate) in 0.2M $NaHCO_3$ buffer. The reaction mixture is passed through a Sephadex G-25 column, and the PNA oligomer fraction is concentrated and treated with 200 mM hydroxylamine hydrochloride solution in water (1 ml). The resulting PNA oligomer (SEQ ID NO:78) H_2N-CTG TCT CCA* TCC TCT TCA CT-COOH has a CH_2-O-$(CH_2)_5$-N-C(O)-CH_2-SH group attached to the C-1 position of the 2-aminoethyl portion of the designated (*) monomer in the oligomer.

B. PNA Oligomer SEQ ID NO:79

PNA oligomer (SEQ ID NO:64) H_2N-CTG TCT CCA*** TCC TCT TCA*** CT-COOH wherein *** represents a pentyl N-phthaloyl oxymethyl group attached at the serine O- portion of the designated monomer in the oligomer, is treated with hydrazine/ethanol followed by 5 mg SATA (N-succinimidyl-S-acetylthioacetate) in

5   0.2M NaHCO$_3$ buffer. The reaction mixture is passed through a Sephadex G-25 column, and the PNA oligomer fraction is concentrated and treated with 200 mM hydroxylamine hydrochloride solution in water (1 ml) to give PNA oligomer (SEQ ID NO:79) H_2N-CTG TCT CCA*** TCC TCT TCA*** CT-COOH having a CH_2-O-$(CH_2)_5$-N-C(O)-CH_2-SH group attached to the serine O- portion of the designated (***) monomer

10   in the oligomer.

EXAMPLE 84

Conjugation of *o*-Phenanthroline to PNA Oligomers

A.   PNA Oligomer SEQ ID NO:80

15   PNA oligomer (SEQ ID NO:78) H_2N-CTG TCT CCA* TCC TCT TCA CT-COOH from Example 83(A) is treated with 2 mg of 5-(iodoacetamide)-*o*-phenanthroline reagent followed by shaking overnight. The oligomer is purified by a size exclusion column and reverse phase HPLC to yield;

             CTG TCT CCA* TCC TCT TCA CT-COOH PNA Oligomer SEQ ID NO:80

20   wherein * represents a PNA monomer functionalized with phenanthroline via a thiol linker of the structure -CH_2-O-$(CH_2)_5$-NH-C(=O)-CH_2-S-CH_2-C(=O)-NH- at the C-1 position of the 2-aminoethyl portion of the designated monomer in the oligomer.

B.   PNA Oligomer SEQ ID NO:81

             PNA oligomer (SEQ ID NO:79) H_2N-CTG TCT CCA*** TCC TCT TCA*** CT-

25   COOH from Example 83B having a CH_2-O-$(CH_2)_5$-N-C(O)-CH_2-SH group attached to the serine O- portion of the designated (***) monomer in the oligomer is treated with 2 mg of 5-(iodoacetamide)-*o*-phenanthroline reagent followed by shaking overnight. The oligomer is purified by a size exclusion column and reverse phase HPLC to yield

             H_2N-CTG TCT CCA*** TCC TCT TCA*** CT-COOH PNA oligomer SEQ ID

30   NO:81

wherein *** represents a PNA monomer functionalized at the serine O- position with phenanthroline via a thiol linker of the structure -CH_2-O-$(CH_2)_5$-NH-C(=O)-CH_2-S-CH_2-C(=O)-NH-.

5   **EXAMPLE 85**

Conjugation of Pyrene to PNA Oligomers

A.   **Single site modification**

PNA oligomer (SEQ ID NO:63) H_2N-CTG TCT CCA* TCC TCT TCA CT-COOH, where * denotes a pentyl N-phthaloyl oxymethyl group at the C-1 position of the

10   2-aminoethyl portion of the designated monomer, (approximately 60 nmols) is treated with hydrazine/ethanol to give the free amine. The free amine is dried in a microfuge tube and is dissolved in 200 µl of 0.2 M $NaHCO_3$ buffer. Pyrene-1-butyric acid N-hydroxysuccinimide ester (*i.e.,* succinimidyl-1-pyrene butyrate, 3 mg, 7.79 µmols, Molecular Probes, Eugene, Oregon) is added followed by 400 µl of DMF. The mixture

15   is incubated at 37°C overnight, and the solution is applied to a Sephadex G-25 column (1 x 40 cm). The PNA oligomer fractions are combined, and the product is purified by HPLC to give PNA oligomer (SEQ ID NO:82) H_2N-CTG TCT CCA* TCC TCT TCA CT-COOH, where * denotes a pyrene attached via a CH_2-O-$(CH_2)_5$-NHC(=O)-$(CH_2)_3$-linking group at the C-1 position of the 2-aminoethyl portion of the designated

20   monomer.

B. **Multiple site modifications**

PNA oligomer (SEQ ID NO:64) H_2N-CTG TCT CCA*** TCC TCT TCA*** CT-COOH, where *** denotes a pentyl N-phthaloyl oxymethyl group at the serine -O portion of the designated monomer, is treated with hydrazine/ethanol to give the free

25   amine. The free amini is further treated with two equivalents of pyrene-1-butyric acid N-hydroxysuccinimide (6 mg in 400 µl DMF) and worked up in the same fashion as Example **85(A)**. Sephadex G-25 purification followed by HPLC purification gives the doubly pyrene-conjugated PNA oligomer SEQ ID NO:83 H_2N-CTG TCT CCA*** TCC TCT TCA*** CT-COOH where *** denotes a pyrene attached via a CH_2-O-$(CH_2)_5$-

30   NHC(=O)-$(CH_2)_3$- linking group at the serine -O portion of the designated monomers in

the oligomer. The oligomer is further purified as per the procedures of **85(A)**.

EXAMPLE 86

Conjugation of Acridine to PNA Oligomers

5   **A.**   **Single site modification**

PNA oligomer SEQ ID NO:63 (2 μmol) is treated with hydrazine/ethanol to give

the free amine. The free amine is dried and dissolved in 1M $NaHCO_3/Na_2CO_3$ buffer,

pH 9.0, 200 μl. 9-Acridinyl-isothiocyanate, (5 mg, 2.1 μmols, Molecular Probes,

Eugene, Oregon) is dissolved in 200 μl DMF. This solution is added to the PNA

10   oligomer, and the mixture is vortexed, covered with aluminum foil and left at 37°C

overnight. The reaction mixture is purified by passing through a Sephadex G-25 column

(1 x 40 cm), concentrated and further purified by HPLC to give PNA oligomer SEQ ID

NO:84 H_2N-CTG TCT CCA* TCC TCT TCA CT-COOH where * denotes a 9-acridinyl

group attached via a CH_2-O-$(CH_2)_5$-NHSCN-linking moiety to the C-1 position of the 2-

15   aminoethyl portion of the indicated (*) monomer.

B. Multiple site modifications

PNA oligomer SEQ ID NO:64 H_2N-CTG TCT CCA*** TCC TCT TCA*** CT-

COOH is treated with hydrazine/ethanol to give the free amine. The free amine is

treated with 400 μl of 1M $Na_2CO_3/NaHCO_3$ buffer (pH 9.0) and further treated with 10

20   mg of 9-acridinyl-isothiocyanate in 400 μl of DMF. The reaction mixture is vortexed,

covered with aluminum foil and left at 37°C overnight. The reaction mixture is purified

as for the single site reaction of Example **85(A)**. The doubly conjugated acridine-

oligonucleotide elutes as the last peak in the HPLC following single-modification

products to give PNA oligomer SEQ ID NO:85 H_2N-CTG TCT CCA*** TCC TCT

25   TCA*** CT-COOH where *** denotes a 9-acridinyl group attached via a CH_2-O-$(CH_2)_5$-

NHSCN-linking moiety to the serine O- position on the designated monomers in the

oligomer.

EXAMPLE 87

30   Conjugation of Porphyrin to PNA Oligomers

Methylpyroporphyrin XX1 ethyl ester (Aldrich) is condensed with aminocaproic

acid using N-hydroxysuccinimide and EDAC. The resultant carboxylic acid is then

activated again with N-hydroxy succinimide and EDAC and treated with PNA oligomer

SEQ ID NO:63 according to the procedure of Example **85(A)** to give PNA oligomer

5   SEQ ID NO:86 H$_2$N-CTG TCT CCA* TCC TCT TCA CT-COOH wherein * denotes a

2-porphyrin group tethered to the C-1 position of the 2-aminoethyl portion of the

designated PNA oligomer.

EXAMPLE 88

10   **Conjugation of Hybrid Intercalator-Ligand to PNA Oligomers**

A.   Photonuclease/intercalator ligand

The photonuclease/intercalator ligand 6-[[[9-[[6-(4-

nitrobenzamido)hexyl]amino]acridin-4-yl]carbonyl]amino]hexanoyl-pentafluorophenyl

ester is synthesized according to the procedure of Egholm et al., *J. Am. Chem. Soc.*

15   **1992**, *114*, 1895.

B.   Single site modification

10 O.D. units of PNA oligomer SEQ ID NO: 63 is treated with hydrazine/ethanol

to deblock the amino group. The resultant material is dissolved in 100 μl of 0.1 M

borate buffer (pH 8.4) and treated with 330 μl of DMF solution (10 mg in 1 ml of DMF)

20   of 6-[[[9-[[6-(4-nitrobenzamido)hexyl]amino]acridin-4-yl]carbonyl]amino]hexanoyl-

pentafluorophenyl ester. The solution is covered with aluminum foil and allowed to

react overnight. The reaction mixture is purified by Sephadex G-25 and HPLC

purification to give PNA oligomer SEQ ID NO:87 H$_2$N-CTG TCT CCA* TCC TCT

TCA CT-COOH wherein * denotes a photonuclease/intercalator ligand attached with a

25   tether to the C-1 position of the 2-aminoethyl portion of the designated monomer in the

oligomer.

C.   Multiple site modification

10 O.D. units A$_{260}$ of PNA oligomer SEQ ID NO:64 is treated with

hydrazine/ethanol to deblock the protected amino positions. The resultant material is

30   dissolved in 200 μl of 0.1 M borate buffer (pH 8.4) and treated with 660 μl of the DMF

solution of 6-[[[9-[[6-(4-nitrobenzamido)hexyl]amino]acridin-4-yl]carbonyl]amino]-
hexanoylpentafluorophenyl ester (10 mg in 1 ml solution) and the solution is covered
with aluminum foil and left aside overnight. The solution is purified by Sephadex G-25
and reverse phase HPLC to give PNA oligomer SEQ ID NO:88 H_2N-CTG TCT CCA***

5   TCC TCT TCA*** CT-COOH wherein *** denotes a photonuclease/intercalator ligand
attached with a tether to the serine O- position of the designated monomers in the
oligomer.

EXAMPLE 89

10   **Conjugation of Bipyridine Complex to PNA Oligomers**

       A.   Bipyridine complex

       Succinimidyl-4-carboxyl-4'-methyl-2,2'-bipyridine is synthesized according to
the procedure of Telser, et al., J. Am. Chem. Soc. **1989**, 111, 7221.

       B.   Single site Modification

15   10 O.D. A_{260} units of PNA oligomer SEQ ID NO:63 is treated with
hydrazine/ethanol to deblock the protected amino group. The resultant material is
reacted with a 200 fold molar excess of succinimidyl-4-carboxyl-4'-methyl-2,2'-
bipyridine in 0.1 M borate buffer pH 8.5/DMF. The solution is purified by Sephadex G-
25 and reverse phase HPLC to yield PNA oligomer SEQ ID NO:89 H_2N-CTG TCT

20   CCA* TCC TCT TCA CT-COOHH$_2$N-CTG TCT wherein * denotes a bipyridinyl
complex attached via a linker to the C-1 position of the 2-aminoethyl portion of the
designated monomer in the oligomer.

EXAMPLE 90

25   **Conjugation of Aryl Azide Photocrosslinkers to PNA Oligomers**

       A.   Conjugation of N-hydroxysuccinimidyl-4-azidobenzoate (HSAB)

       PNA oligomer SEQ ID NO:63 is treated with hydrazine/ ethanol to give the free-
amine containing PNA oligomer:

       CTG TCT CCA* TCC TCT TCA CT-COOH SEQ ID NO:90

30   wherein * denotes a pentylamino oxymethyl group attached at the C-1 of the 2-

aminoethyl portion of the designated monomer in the oligomer.

PNA oligomer SEQ ID NO:90 (approx. 50 nmols) is dried, dissolved in 500 ml of 0.2M NaHCO$_3$ buffer pH 8.1 and treated with 25 mg of N-hydroxysuccinimidyl-4-azidobenzoate (HSAB, 96 μmols, available both from Pierce & Sigma) dissolved in 500

5 μl of DMF. The mixture is stirred overnight at 37°C and passed twice over Sephadex G-25 column (1 x 40 cm). The PNA oligomer fraction is purified by reverse-phase HPLC (5% → 40% CH$_3$CN in 60 min) on a reverse phase column to give PNA oligomer SEQ ID NO:91 CTG TCT CCA* TCC TCT TCA CT-COOH wherein * denotes an HSAB group attached via a tether to the C-1 position of the 2-aminoethyl portion of the

10 designated monomer in the oligomer.

B.   Conjugation of N-succinimidyl-6(4'-azido-2'-nitrophenyl-amino)hexanoate

PNA oligomer SEQ ID NO:90 is dissolved in 500 ml of NaHCO$_3$ buffer (0.2M, pH 8.1) and treated with 500 μL of DMF containing 500 mg of N-succinimidyl-6(4'-

15 azido-2'-nitrophenyl-amino)hexanoate (SANPAH, 128 μmols, available both from Pierce and Sigma). The reaction vial is wrapped with aluminum foil and heated at 37°C overnight. The reaction mixture is passed twice over a Sephadex G-25 column (1 x 40 cm). The PNA oligomer fraction is purified by reverse-phase HPLC (5% → 40% CH$_3$CN in 60 min.) on a reverse phase column to give PNA oligomer SEQ ID NO:92

20 CTG TCT CCA* TCC TCT TCA CT-COOH wherein * denotes a 6(4'-azido-2'-nitrophenyl-amino)hexanoate group attached via a tether to the C-1 position of the 2-aminoethyl portion of the designated monomer in the oligomer.

EXAMPLE 91

25 **Conjugation of Imidazole-4-acetic acid to PNA Oligomers**

A.   Activated imidazole-4-acetic acid

Imidazole-4-acetic acid was reacted with 2,4-dinitrofluoro benzene. The resulting imidazole-N-(DNP)-4-acetic acid was converted to its N-hydroxy succinimide ester by treating with NHS/EDAC according to the procedure of Example **78**.

30   **B.   PNA conjugation**

10 O.D. A_{260} units of PNA oligomer SEQ ID NO:90 is reacted with a 100 fold

molar excess of imidazole-N-(DNP)-4-acetic acid NHS ester in O.1M borate buffer pH

8.5/DMF (200 µL each). The mixture is stirred overnight and further treated with 200

µL of mercaptoethanol to cleave off the DNP protecting group. The resulting reaction

5   mixture is purified by passing through a Sephadex G-25 column followed by reverse

phase HPLC to give PNA oligomer SEQ ID NO 93 CTG TCT CCA* TCC TCT TCA

CT-COOH wherein * denotes an imidazolyl group attached via a tether to the C-1

position of the 2-aminoethyl portion of the designated monomer in the oligomer.

10   **EXAMPLE 92**

Conjugation of Metal Chelating Agents to PNA Oligomers

A.   EDTA Complex

To form an EDTA Fe(II) complex for coupling to a PNA oligomer as a nucleic

acid cleaving agent, tricylohexyl ester of EDTA is synthesized according to the

15   procedure of Sluka, et al., J. Am. Chem. Soc. **1990**, 112, 6369.

B.   PNA Oligomer Modification

The tricyclohexyl ester of EDTA (1.25 mg, 1.94 mmol) and

hydroxybenzotriazole (HOBt, 1 mg, 6.6 mmol) are dissolved in DMF (50 µL) and

EDAC 10 µL is added. To this solution, PNA oligomer SEQ ID NO:90 (10 OD units) in

20   100 µL 0.1M borate buffer is added and left overnight. The solution is passed through a

Sephadex G-25 column and the PNA oligomer fraction is treated with conc. NH₃ (100

µL) for 1 hr. to cleave off the acid protecting groups. Finally purification is effected by

size exclusion chromatography and HPLC.

C.   DTPA Site Modification

25   PNA oligomer SEQ ID NO:90 is treated with diethylene triamine pentaacetic

anhydride (DTPA) in 0.1M NaHCO₃/DMF to offer single-site modification. The

conjugate is complexed with Gadolinium ion (Gd III) to form a contrasting agent, which

is useful as, inter alia, an uptake measuring agent. The resulting PNA oliogmer SEQ ID

NO:94 CTG TCT CCA* TCC TCT TCA CT-COOH wherein * denotes the complexed

30   Gadolinium ion attached via a tether to the C-1 position of the 2-aminoethyl portion of

the designated monomer in the oligomer.

EXAMPLE 93

Conjugation of Cholesterol To PNA Oligomers

5   **A.   Method 1 - via aminolinker**

Cholesterol-hemisuccinate is first converted to its N-hydroxy succinimide ester, which is then conjugated to PNA oligomer SEQ ID NO:90 according to the procedure of Example **85(A)** to give PNA oligomer SEQ ID NO:95 CTG TCT CCA* TCC TCT TCA CT-COOH wherein * denotes cholesterol attached via a tether to the C-1 position of the 2-aminoethyl portion of the designated monomer in the oligomer or PNA oligomer SEQ ID NO:64 which is first treated with hydrazine/ethanol to free the amine and then further treated as per the procedures of Example **85(A)** to give PNA oligomer SEQ ID NO:96 H_2N-CTG TCT CCA*** TCC TCT TCA*** CT-COOH wherein *** denotes a cholesterol attached via a tether to the serine O- position of the designated monomers in the oligomer.

B. Method 2 - Conjugation of Cholesterol via a Disulfide Bridge

1.   S-(2-thio-5-nitropyridyl)-thio cholesterol

Thiocholesterol (1.4 g, 3.5 mmol) is added to a stirred solution of 2,2'-dithiobis(5-nitropyridine) (1.4 g 4 mmol) in chloroform (20 mL) containing glacial acetic acid (400 µL) under an argon atmosphere. The reaction is allowed to continue overnight at room temperature, after which the precipitated 5-nitropyridine-2-thione is removed and the solution evaporated and purified on a silica column to give S-(2-thio-5-nitropyridyl)-thio cholesterol.

2.   Conjugation to PNA Oligomer

PNA oligomer SEQ ID NO:59 H_2N-GCA*T-COOH, wherein * denotes a disulfide protected thiohexyloxymethyl linking group attached to the C-1 position of the 2-aminoethyl portion of the monomer in the oligomer, is reacted with an excess of S-(2-thio-5-nitropyridyl)-thiocholesterol to attach the cholesterol moiety to the thiol group of the PNA oligomer via a disulfide bridge to give PNA oligomer SEQ ID NO:97 H_2N-GCA*T-COOH wherein * denotes a cholesterol group attached via a tether to the C-1

position of the 2-aminoethyl portion of the monomer in the oligomer.

Example 94

Coupling of Conjugate Groups to Primary Amino Groups of a PNA or a PNA

Derivative

PNAs and PNA derivatives having primary amino groups can have a conjugate group attached thereto including terminal amine groups and those "masked" during PNA oligomer synthesis and later regenerated.

Amino hexanoic acid (AHA) groups are added to PNA oligomers during solid phase synthesis as N-t-butyloxycarbonyl-ε-amino-hexanoic acid following the standard protocols.

     I.   **General Synthesis of PNA Conjugates via an Amino Group**

     A.   **Coupling to Primary Amino Groups of H-PNA-NH$_2$, H-PNA-Lys-NH$_2$ and AHA-PNA-NH$_2$ (AHA = -NH-(CH$_2$)$_5$-C=(O)O-)**

The three 2-aminoethylglycine-based PNA structures below were synthesized such that each has at least one free amino group:

5   where B is a nucleobase or a derivative thereof.

B.   Coupling of D-biotin-N-hydroxysuccinimide Ester (BAE)

H-PNA-NH$_2$, H-PNA-Lys-NH$_2$, or AHA-PNA-NH$_2$ (0.2μL, 2.5 μg/μL in DMF)

is added to a reaction flask containing 0.2M Na$_2$CO$_3$ (2.5 μL), 0.2M NaHCO$_3$ (2.5 μL),

and CH$_3$CN (5 μL). BAE (0.2μL, 20 μg/μL in DMF) is added to the reaction flask in

10   three portions, while shaking the flask. The reaction is allowed to proceed at room

temperature for 15 minutes after each addition. Fifteen minutes after the last addition

the reaction mixture is separated by HPLC (reverse phase C-18 column) using the

following two-buffer gradient system:

15   Time (min)   % Buffer A   % Buffer B

0	100	0
10	100-r	r
40	100-q	q
50	100	0

5

Buffer A = 0.1% TFA in H_2O.

Buffer B = 0.1% TFA; 60% CH_3CN; 40% H_2O

C. Coupling of Acridine-N-hydroxysuccinimide Ester (AAE)

H-PNA-NH_2, H-PNA-Lys-NH_2, or AHA-PNA-NH_2 (27.5 μg) is added to a

10   reaction flask containing 0.2M Na_2CO_3 (2.5 μL), 0.2M $NaHCO_3$ (2.5 μL), and CH_3CN

(5 μL). AAE (0.7μL, 20 μg/μL in DMF) is added to the reaction flask in three portions,

while shaking the flask. The reaction is allowed to proceed at room temperature for 15

minutes after each addition. Fifteen minutes after the last addition the reaction mixture

is separated by HPLC according to the procedure of Example **44(B)**.

15   **D.   Coupling of Azidobenzoyl Chloride (ABC)**

H-PNA-NH_2, H-PNA-Lys-NH_2, or AHA-PNA-NH_2 (1.3 μL, 2.5 μg/μL in DMF)

is added to a reaction flask containing 0.2M Na_2CO_3 (10 μL), and CH_3CN (10 μL).

Azidobenzoyl chloride (30μg) is added to the reaction flask, which is allowed to remain

at room temperature for fifteen minutes. Orthophosphoric acid (O.5 μl, 85% solution) is

20   added, and the solution is extracted with chloroform five times. The aqueous phase is

then separated on the HPLC according to the procedure of Example **94(B)**.

E.   Coupling of Alkanoyl Chloride

H-PNA-NH_2, H-PNA-Lys-NH_2, or AHA-PNA-NH_2 (1.3 μL, 2.5 μg/μL DMF) is

added to a reaction flask containing 0.2M Na_2CO_3 (2.5 μL), $NaHCO_3$ (2.5 μL), and

25   CH_3CN (5 μL). One microliter of the alkanoyl chloride is added to the reaction flask

with shaking, and the reaction is allowed to proceed at 37°C for 15 minutes. The

aqueous solution is extracted with hexane three times, and the resulting aqueous phase is

separated by HPLC according to the procedure of Example **94(B)**.

F.   Coupling of Cholesteryl Chloroformate (CC)

30   H-PNA-NH_2, H-PNA-Lys-NH_2, or AHA-PNA-NH_2 (1.3 μL, 2.5 μg/μL in DMF)

is added to a reaction flask containing 0.2M Na_2CO_3 (2.5 μL), and $CHCl_3$ (2.5 μL). Cholesteryl chloroformate (30 μg) is added to the reaction flask, which is shaken at room temperature for five days and nights. The aqueous phase is then separated on the HPLC according to the procedure of Example **94(B)**.

G.   Coupling of Dansyl Chloride (DC)

Dansyl chloride (0.16 μL of a 1 μg/μL in CH_3CN) is added to a reaction flask containing 0.2M Na_2CO_3 (0.5 μL) and 0.2M $NaHCO_3$ (0.5 μL). H-PNA-NH$_2$, H-PNA-Lys-NH$_2$, or AHA-PNA-NH$_2$ (3.34 μL, 2.5 μg/μL DMF) is then added, and the reaction is allowed to proceed in the dark at room temperature for 1.5 hours. The reaction mixture is then separated by HPLC according to the procedure of Example **94(B)**.

H.   Coupling of Folic Acid (FA)

H-PNA-NH$_2$, H-PNA-Lys-NH$_2$, or AHA-PNA-NH$_2$ (1.3 μL, 2.5 μg/μL DMF) is placed in a reaction flask containing folic acid (0.5 μL of 1μg/μL in DMSO), EDC (0.16 μL, 10 μg/μL in DMSO) and DMSO (3.34 μL). The reaction is allowed to proceed overnight in the dark at room temperature, and then folic acid (0.5 μL, 1μg/μL in DMSO) and EDC (0.16 μL, 10 μg/μL in DMSO) are added to the flask. The reaction is allowed to proceed another six hours in the dark at room temperature, followed by another addition of folic acid (0.5 μL, 1μg/μL in DMSO) and EDC (0.16 μL, 10 μg/μL in DMSO). The reaction is allowed to continue overnight in the dark at room temperature. Water (16.26 μL) is added, and the reaction mixture is lyophilized, taken up in water, and separated by HPLC according to the procedure of Example **94(B)**.

I.   Coupling of Maleimido Active Ester (N-Maleimidopropionic Acid N-hydroxysuccinide Ester) (MAE)

H-PNA-NH$_2$, H-PNA-Lys-NH$_2$, or AHA-PNA-NH$_2$ (1.3 μL, 2.5 μg/μL in DMF) is added to a reaction flask containing 0.2M Na_2CO_3 (1.25 μL), 0.2M $NaHCO_3$ (1.25 μL), and CH_3CN (6.9 μL). MAE (0.2 μL, 20 μg/μL in DMF) is added to the reaction flask in three portions, while shaking the flask. The reaction is allowed to proceed at room temperature for 15 minutes after each addition. Fifteen minutes after the last addition the reaction mixture is separated by HPLC according to the procedure of Example **94(B)** except that TFA is omitted from the gradient, and the product is

collected.

J.   Coupling of Quinone active ester (anthraquinone-2 carboxylic acid-ODhbt ester) (QAE)

H-PNA-NH$_2$, H-PNA-Lys-NH$_2$, or AHA-PNA-NH$_2$ (1.3 μL, 2.5 μg/μL in DMF) is added to a reaction flask containing 0.2M Na$_2$CO$_3$ (1.3 μL), 0.2M NaHCO$_3$ (1.3 μL), and CH$_3$CN (5.2 μL). QAE (0.9 μL, 5 μg/μL in DMF) is added to the reaction flask in three portions, while shaking the flask. The reaction was allowed to proceed at room temperature for 15 minutes after each addition. Fifteen minutes after the last addition the reaction mixture is separated by HPLC according to the procedure of Example 94(B).

K.   Coupling of Fluorescein

A PNA or PNA derivative having a single free NH$_2$ such as H$_2$N-GCAT-COOH is dissolved in tetrahydrofuran:water to provide a solution that is 0.1 M in PNA and to this is added fluorescein isothiocyanate to give a solution which is 0.1-1.0 M in fluorescein isothiocyanate. The solution is stirred for 0.1-2 hours, then evaporated to a solid and purified by preparative HPLC.

L.   Coupling of EDTA

EDTA conjugates are prepared from EDTA-tribenzyl ester by standard solid phase synthesis. Purification is by HPLC.

M.   Coupling of Nitrillo Triacetic Acid (NTA)

NTA conjugates are prepared as dibenzylesters from glycine-*tert*-butylester using standard solid phase synthesis. Purification is by HPLC.

N.   Coupling of 5-Amino-1,10-phenanthroline-glutaramide (Phen$_1$)

1.   5-Amino-1,10-phenanthroline-glutarimide

5-Amino-1,10-phenanthroline (350mg, 1.8mmol) was refluxed with glutaric anhydride (2.0g, 17.5mmol) in pyridine (3 mL) and triethylamine (3 mL) for 2 hours. The reaction mixture was reduced to a viscous oil by rotary evaporation. Column purification on silica with 10% methanol in methylene chloride (product produced bright red spots with Fe(II) on TLC) gave the crude product (320mg 1.1mmol). Further

purification of the crude product was by recrystalization once from 30 mL boiling water to give the title compound (250mg, 0.85mmol) as centimeter long transparent needles.

2.   5-Amino-1,10-phenanthroline-glutaramide

The purified title compound from **94(I)N(1)** above (250mg, 0.85mmol) was refluxed in water (3 mL) and triethylamine (3 mL) for 10 minutes. The material was evaporated on a rotary evaporator and further coevaporated with 3, 10 mL portions of water. This afforded the title compound in a quantitative yield with a trace of triethylamine as a contaminant. The material was recrystalized from water (30 mL) to give after drying the purified title compound (200mg, 0.61mmol) (72%) as a white macrocrysaline monohydrate.

3.   Coupling to PNA

The triethylammonium salt of 5-Amino-1,10-phenanthroline-glutaramide (60mg, 0.14mmol) from Example **94(I)N(2)** above was dissolved in DMF (1 mL) and pyridine (0.5 mL). HBTU (45 mg, 0.12mmol) was added followed by diisopropylethylamine (0.05 mL, 0.3mmol). This solution is then added to an N-terminal end of a growing PNA or can be used to functionalize gel.

O.   Coupling of Piperazine-N-acetic acid-N'-acetic acid-(5'-phenanthryl)-amide (Phen$_2$)

1.   Piperazine-N-acetic acid-*tert*-butylester-N'-acetic acid-(5'-phenanthryl)-amide

Iodoacetamido-phenanthroline (150mg, 0.41mmol) and piperazineacetic acid-
tert-butylester (90mg, 0.48mmol) were mixed in DMF (1 mL) and triethylamine (0.5
mL). The reagents were stirred for 1 hour at room temperature and then the solvents
were removed by rotary evaporator. The product was purified by silica gel column

5   chromatography with pyridine (15%) in DMF as the eluent (product produced bright red
spots with Fe(II) on TLC) to give the title compound in a 70% yield (120mg,
0.29mmol).

2.   Piperazine-N-acetic acid-N'-acetic acid-(5'-phenanthryl)-amide

Piperazine-N-acetic acid-*tert*-butylester-N'-acetic acid-(5'-phenanthryl)-amide

10 (120mg, 0.29mmol) was dissolved in dichloromethane (3 mL) and trifluoroacetic acid (3
mL). The mixture was stirred at room temperature for 3 hours and then the solvent was
removed in *vacuo* at 0.1 mmHg. This gave the title compound (with traces of starting
material) in a quantitative yield as a trifluoroacetate.

3.   Coupling to PNA

15 Piperazine-N-acetic acid-N'-acetic acid-(5'-phenanthryl)-amide (150mg,
0.25mmol) as a trifluoroacetate was dissolved in DMF (2mL) and diisopropylethylamine
(1mL). To the resulting solution (1.5mL) was added HBTU (38mg, 0.1mmol) and this
solution is then added to an N-terminal end of a growing PNA or can be used to
functionalize gel.

20 ### P.   Coupling of Fluor$_{phet}$ and Fluor$_{met}$

1.   Fluorescein-(2-phenyl)ethylester-O-acetic acid-*tert*-butylester

Fluorescein-(2-phenyl)ethylester (3g, 6.9mmol) was dissolved in DMF (10mL)
and triethylamine (2.5mL). The mixture was heated to 70 °C, and bromoacetic acid *tert*-
butylester (2g, 10mmol) was added. The reaction mixture was maintained at 70 °C for 2

25 hours and then cooled to room temperature. Dichloromethane (40ml) was added and the
resulting solution was extracted with 5% NaHCO$_3$ (2 X 10 mL). The organic phase was
dried over Na$_2$CO$_3$, filtered and evaporated in *vacuo*. The residue was purified twice
using silica gel column chromatography with methanol (3-15%) in dichloromethane as
the eluent. The title was obtained in a 53% yield (2.0g, 3.6mmol).

30 ### 2.   Fluorescein-(2-phenyl)ethylester-O-acetic acid

Fluorescein-(2-phenyl)ethylester-O-acetic acid-*tert*-butylester (2.0g, 3.6mmol) was dissolved in dichloromethane (10ml) and trifluoroacetic acid (10 mL). The mixture was stirred overnight and then evaporated to a viscous oil. Following 2 column purifications with methanol (10%) in dichloromethane as the eluent the title compound precipitated from the last eluate, and was dissolved in warm ethanol (96%, 150 mL). The ethanol was evaporated to leave approximately 30 mL and upon cooling the title compound precipitated. The title compound was isolated as a microcrystaline red powder in a 78% yield (1.4g, 2.8mmol).

3.   Fluorescein-monomer-ethylester

Fluorescein-(2-phenyl)ethylester-O-acetic acid (1.08g, 2.0mmol) and Compound 8, N-Boc-aminoethyl glycine ethyl ester (500mg, 2.0mmol) was dissolved in DMF (15 mL) and dichloromethane (5mL), HDBTU (324mg) and DCC (480mg) were added. The mixture was heated to 50 °C for 1 hour and then allowed to stand at room temperature overnight. DCU was filtered off and ether (50 mL) was added followed by washing with 5% $NaHCO_3$ (20 mL) and 5% $NaHSO_4$ (20 mL). The organic phase was dried and evaporated to dryness. Purification was by silica gel column chromatography using methanol (0-15%) in dichloromethane as eluent. The title compound was isolated in a 67% yield (0.98g, 13.5mmol).

4.   Fluorescein-monomers $Fluor_{phet}$ and $Fluor_{met}$

Fluorescein-(2-phenyl)ethylester (300mg, 41mmol) was dissolved in THF (20mL) and LiOH (1 M, 5 mL) was added. The mixture was stirred for 35 minutes at room temperature, poured onto ice/dilute $NaHSO_4$ and was extracted with ethyl acetate. The organic phase was dried and evaporated to dryness. Purification was by silica gel column chromatography using DMF as eluent. The title compound $Fluor_{phet}$ was isolated as a dark red oil in a 30% yield (100mg, 1.4mmol). The monomers are incorporated into PNA oligomers using standard protocols. (The transesterification product $Fluor_{met}$ was also observed in one synthesis and used to form a PNA conjugate.)

Q.   PNA-Staphylococcal Nuclease Conjugate

Maleimido-PNA derivative (3µg) from **94(I)** above was dissolved in 10mM $NaPO_4$ buffer (100 µL, pH 7.0) and was incubated with SH-straphylococcal nuclease

mutant Lys116-Cys (10 µg) (Corey, *et.al., Science,* **1987**, *238,* 1401), for 2 hours at room temperature. The PNA-nuclease conjugate was separated from excess maleimido-PNA by chromatography on a

Sephadex G-50 in 10 mM $NaPO_4$ pH 7.0.

II.   Coupling of Conjugates to AHA-$T_2CT_2CT_4$-NH_2

A.   Acridine-AHA-$T_2CT_2CT_4$-NH_2

PNA AHA-$T_2CT_2CT_4$-NH_2 (SEQ ID NO:98) was synthesized using standard methods and techniques as per Example **26**. 3300µg of PNA (SEQ ID NO:98) was dissolved in 0.2M Na_2CO_3 (300µL), 0.2M $NaHCO_3$ (300µL) and CH_3CN (600µL). Acridine-N-hydroxy-succinimide-ester (AAE) (100µL, 20µg/µL DMF) was added in 5 portions while shaking the reaction vessel. The mixture was left for 15 minutes at room temperature after each portion of AAE. The reaction was monitored by HPLC using a reversed phase spherisorp S50DS1 C-18 column (250x4.6 mm) and a gradient of varying concentrations of Buffer A (0.1% TFA in H2O) and Buffer B (0.1% TFA, 60% CH_3CN, 40% H_2O). The retention time of the starting material PNA AHA-$T_2CT_2CT_4$-NH_2 (SEQ ID NO:98) was approximately 15 minutes and that of the final PNA, acridine-AHA-$T_2CT_2CT_4$-NH_2 was 23 minutes.

After completion of the reaction, the PNA was precipitated with 12 ml of isobutanol, lyophilized and then redissolved in 1 ml H_2O. The acridine-PNA was purified by HPLC using a reversed phase C-18 column and the same conditions as above.

In a subsequent binding assay of the acridine-PNA to dsDNA the Kd shows that there is a 10-100 fold increase in the binding compared to that of unmodified PNA and that it binds to dsDNA at 150 mM salt at µmolar (6.5) concentrates.

B.   Anthraquinone-AHA-$T_2CT_2CT_4$-NH_2

115µg of PNA AHA-$T_2CT_2CT_4$-NH_2 (SEQ ID NO:98) was dissolved in 0.2M Na_2CO_3 (111µL), 0.2M $NaHCO_3$ (111µL) and CH_3CN (444µL). Anthraquinone-DHBT-ester (QAE) (79.5µL, 5µg/µL CH_3Cl) was added in 3 portions while shaking the reaction vessel. The mixture was left for 10 minutes at room temperature after each portion of QAE was added. The reaction was monitored by HPLC using a reversed phase

spherisorp S50DS1 C-18 column (250x4.6 mm) and a gradient of varrying
concentrations of Buffer A (0.1% TFA in H2O) and Buffer B (0.1% TFA, 60% CH_3CN,
40% H_2O). The retention time of the starting material PNA AHA-$T_2CT_2CT_4$-NH_2 (SEQ
ID NO:98) was approximately 21 minutes and that of the final PNA, acridine-AHA-

5      $T_2CT_2CT_4$-NH_2 was 30 minutes.

After completion of the reaction, the H_2O phase with the PNA was lyophilized
and then redissolved in 1 ml H_2O. The Quinone-PNA was purified by HPLC using a
reversed phase C-18 column and the same conditions as above.

C.   Biotin-AHA-$T_2CT_2CT_4$-NH_2

10   78μL, 2.5μg/μL of PNA AHA-$T_2CT_2CT_4$-NH_2 (SEQ ID NO:98) was dissolved
in 0.2M Na_2CO_3 (150μL), 0.2M $NaHCO_3$ (150μL) and CH_3CN (300μL). Biotin-N-
hydroxy-succinimide-ester (BAE) (120μL, 2μg/μL DMF) was added in 4 portions while
shaking the reaction vessel. The mixture was left for 15 minutes at room temperature
after each portion of BAE was added. The reaction was monitored by HPLC using a

15   reversed phase spherisorp S50DS1 C-18 column (250x4.6 mm) and a gradient of
varrying concentrations of Buffer A (0.1% TFA in H2O) and Buffer B (0.1% TFA, 60%
CH_3CN, 40% H_2O). The retention time of the starting material PNA AHA-$T_2CT_2CT_4$-
NH_2 (SEQ ID NO:98) was approximately 15 minutes and that of the final PNA,
acridine-AHA-$T_2CT_2CT_4$-NH_2 was 41 minutes.

20   After completion of the reaction, the H_2O phase with the PNA was lyophilized
and then redissolved in 0.45 ml H_2O. The biotin-PNA was purified by HPLC using a
reversed phase C-18 column and the same conditions as above.

D.   Folic Acid-AHA-$T_2CT_2CT_4$-NH_2

400μL 2.5μg/μL of PNA AHA-$T_2CT_2CT_4$-NH_2 (SEQ ID NO:98) was mixed

25   with 154μL 1.0μg/μL DMSO folic acid, 50μL 10μg/μL of DMSO EDC, and 1228μL
DMSO. The mixture was left overnight in the dark at room temperature. The next day
150μL 1.0μg/μL DMSO folic acid plus 50μL 10μg/μL DMSO EDC was added and the
mixture was left for 6 hours in the dark at room temperature. Then 150μL 1.0μg/μL
DMSO folic acid plus 50μL 10μg/H_2O DMSO EDC was added and the mixture was left

30   overnight in the dark at room temperature. The following day H_2O (5mL) was added

and the H_2O-phase was lyophilized and then redissolved in 400μL 1:1 mixture of H_2O and DMSO. The reaction was monitored by HPLC using a reversed phase spherisorp S50DS1 C-18 column (250x4.6 mm) and a gradient of varrying concentrations of Buffer A (0.1% TFA in H2O) and Buffer B (0.1% TFA, 60% CH_3CN, 40% H_2O). The

5   retention time of the starting material PNA AHA-$T_2CT_2CT_4$-NH (SEQ ID NO:98) was approximately 24 minutes and that of the final PNA, folic acid-AHA-$T_2CT_2CT_4$-NH was 34 minutes.

The folic acid PNA was purified by HPLC using a reversed phase C-18 column and the same conditions as above.

10   **E.   Octanoyl-AHA-$T_2CT_2CT_4$-NH_2**

370μL, 2.0μg/μL of PNA AHA-$T_2CT_2CT_4$-NH_2 (SEQ ID NO:98) was dissolved in 0.2M Na_2CO_3 (340μL), 0.2M $NaHCO_3$ (340μL) and CH_3CN (680μL). 80μL $CH_3(CH_2)_6COCl$ was added in 4 portions while shaking the reaction vessel. The mixture was left for 15 minutes at room temperature after each portion of

15    $CH_3(CH_2)_6COCl$ was added. The reaction was monitored by HPLC using a reversed phase spherisorp S50DS1 C-18 column (250x4.6 mm) and a gradient of varrying concentrations of Buffer A (0.1% TFA in H2O) and Buffer B (0.1% TFA, 60% CH_3CN, 40% H_2O). The retention time of the starting material PNA AHA-$T_2CT_2CT_4$-NH (SEQ ID NO:98) was approximately 21 minutes and that of the final PNA, acridine-AHA-

20    $T_2CT_2CT_4$-NH was 30 minutes.

After completion of the reaction, the H_2O phase with the PNA was lyophilized and then redissolved in 0.45 ml H_2O. The octanoyl-PNA was purified by HPLC using a reversed phase C-18 column and the same conditions as above.

                   F.   Psoralen-AHA-$T_2CT_2CT_4$-NH_2

25   4600μg of PNA AHA-$T_2CT_2CT_4$-NH (SEQ ID NO:98) was dissolved in H_2O (920μL), 0.2M $NaHCO_3$ (230μL), and 1917μL DMF. 552μL 10μg/μL DMF psoralene-N-hydroxy-succinimide-ester (PAE) was added in 4 portions while shaking the reaction vessel. The mixture was left for 15 minutes at room temperature after each portion of PAE was added. The reaction was monitored by HPLC using a reversed phase

30   spherisorp S50DS1 C-18 column (250x4.6 mm) and a gradient of varrying

concentrations of Buffer A (0.1% TFA in H2O) and Buffer B (0.1% TFA, 60% CH$_3$CN, 40% H$_2$O). The retention time of the starting material PNA AHA-T$_2$CT$_2$CT$_4$-NH (SEQ ID NO:98) was approximately 20 minutes and that of the final PNA, psoralen-AHA-T$_2$CT$_2$CT$_4$-NH was 30 minutes.

5   After completion of the reaction, the H$_2$O phase with the PNA was lyophilized and then redissolved in 1 mL H$_2$O. The psoralen-PNA was purified by HPLC using a reversed phase C-18 column and the same conditions as above

	(SEQ ID NO:99)	Acridine-AHA-T$_2$CT$_2$CT$_4$-NH$_2$
10	(SEQ ID NO:100)	AnthraQuinone-AHA-TTC TTC TTT T-NH2
	(SEQ ID NO:101)	Biotin-AHA-TTC TTC TTT T-NH2
	(SEQ ID NO:102)	Folic acid-AHA-TTC TTC TTT T-NH2
	(SEQ ID NO:103)	Octanoyl-AHA-TTC TTC TTT T-NH2
	(SEQ ID NO:104)	Psoralen-AHA-T$_2$CT$_2$CT$_4$-NH$_2$
15	(SEQ ID NO:105)	H-TTT TTT TTT T-Lys-Biotin
	(SEQ ID NO:106)	Biotin-H-TTT TTT TTT T-Lys-Biotin
	(SEQ ID NO:107)	H-TTT TTT TTT T-Lys-Hexyl-Biotin
	(SEQ ID NO:108)	Biotin-AHA-Tyr-TTC TTC TTT T-Lys-NH$_2$
	(SEQ ID NO:109)	Biotin-AHA-CTC TTT TTT T-NH$_2$
20	(SEQ ID NO:110)	Biotin-TTT CTT CTC ACT TCT T-NH$_2$
	(SEQ ID NO:111)	Biotin-Hexyl-TTT CTT CTC ACT TCT T-NH$_2$
	(SEQ ID NO:112)	H-TTT TTT TTT T-Lys-Maleimide
	(SEQ ID NO:113)	Maleimide-H-TTT TTT TTT T-Lys-Maleimide
	(SEQ ID NO:114)	H-Tyr-TTT TTT TTT T-Lys-Maleimide
25	(SEQ ID NO:115)	Maleimide-AHA-TTC TTC TTT T-NH$_2$
	(SEQ ID NO:116)	Maleimide-AHA-Tyr-TTC TTC TTT T-NH$_2$
	(SEQ ID NO:117)	Maleimide-TTT TT-NH$_2$
	(SEQ ID NO:118)	Quinone-AHA-TTC TTC TTT T-NH$_2$
	(SEQ ID NO:119)	Quinone-AHA-Tyr-TTC TTC TTT T-NH$_2$
30	(SEQ ID NO:120)	Acridine-AHA-TTC TTC TTT T-NH$_2$

(SEQ ID NO:121)   Acridine-AHA-TTT TGG TGT GGG TCT-NH$_2$

(SEQ ID NO:122)   N$_3$-Benzoyl-AHA-TTC TTC TTT T-NH$_2$

(SEQ ID NO:123)   N$_3$-Benzoyl-TTT CTT CTC ACT TCT T-NH$_2$

(SEQ ID NO:124)   H-GTA GAT CAC T-Lys-Benzoyl-N$_3$

5   (SEQ ID NO:125)   H-AGT CAT CTA C-Lys-Benzoyl-N$_3$

(SEQ ID NO:126)   Dodecanoyl-AHA-TTC TTC TTT T-NH$_2$

(SEQ ID NO:127)   Dodecanoyl-AHA-CTC TTT TTT T-NH$_2$

(SEQ ID NO:128)   Myristoyl-AHA-CTC TTT TTT T-NH$_2$

(SEQ ID NO:129)   Cholesteryl-formyl-AHA-TTC TTC TTT T-NH$_2$

10   (SEQ ID NO:130)   Cholic Acid-FLUO$_{phet}$-CTC TTT TTT T-NH$_2$

(SEQ ID NO:131)   Dansyl-TTT TT-NH$_2$

(SEQ ID NO:132)   PHEN$_1$-AHA-TGT ACG TCA CAA CTA-NH$_2$

(SEQ ID NO:133)   EDTA-AHA-TGT ACG TCA CAA CTA-NH$_2$

(SEQ ID NO:134)   EDTA-AHA-TTC TTC TTT T-NH$_2$

15   (SEQ ID NO:135)   EDTA-AHA-Lys$_2$-TTC TTC TTT T-Lys$_2$NH$_2$

(SEQ ID NO:136)   PHEN$_2$-AHA-TTC TTC TTT T-NH$_2$

(SEQ ID NO:137)   PHEN$_2$-AHA-Lys-Lys-TTC TTC TTT T-NH$_2$

(SEQ ID NO:138)   NTA-AHA-TTC TTC TTT T-NH$_2$

(SEQ ID NO:139)   NTA-AHA-Lys$_2$-TTC TTC TTT T-Lys$_2$-NH$_2$

20   (SEQ ID NO:140)   FLUO$_{met}$-AHA-CTC TTT TTT T-NH$_2$

where Lys is lysine, Tyr is tyrosine, AHA is amino hexanoic acid, NTA is nitrillo

triacetic acid, PHEN$_1$ is a substituted phenathroline as reported by Jenkins, Y. and

Barton, J.K., *JACS* **1992**, *114*, 8736-38. PHEN$_2$ is a similar compound that incorporates

a piperazine linker. FLUO$_{phet}$ is a partially deprotected flourescein monomer while

25   FLUO$_{met}$ is a transeterified flourescein product.

Example 95

N-Terminal Polyamine End Labeled PNA Oligomers

Polyamines are attached to the N-terminal end of PNA oligomer having the

30   sequence H$_2$N-GCA TGC AT-C(O)OCH$_3$ SEQ ID NO:141 synthesized as per standard

methods and techniques, wherein the polyamine is one of the following:

TABLE III

- 1,6 Diaminohexane	PNA Oligomer SEQ ID NO:142
- Diethylenetriamine	PNA Oligomer SEQ ID NO:143
- Triethylenetetramine	PNA Oligomer SEQ ID NO:144
- Spermine	PNA Oligomer SEQ ID NO:145
- Pentaethylenehexamine	PNA Oligomer SEQ ID NO:146

A. Preparation of the PNA Oligomer

PNA oligomer SEQ ID NO:141 having the terminal carboxyl group protected as a methyl ester and having a free amino terminal group is prepared in accordance with Examples **26-28**, and standard protection protocols.

B. Preparation of Polyamine Functionalized PNA Oligomer

The PNA oligomer from Example **95(A)** above is dissolved in freshly prepared $NaHCO_3$ buffer (0.2 M, pH 8.1) and treated with a solution of disuccinimidyl suberate (DSS) (approximately 5 mgs) dissolved in 150 ul of methyl sulfoxide (DMSO). The reaction mixture is left to react for 20 minutes at room temperature. The mixture is then passed over a Sephadex G-25 column (0.7 x 45 cm) to separate the activated PNA oligomer-DSS from the excess DSS. The PNA oligomer-DSS is then frozen immediately and lyophilized to dryness. A solution of polyamine in 0.33 M NaOAc (approximately 6 mg polyamine in 300 ul 0.33 M NaOAc, pH 5.2, final solution pH 6-8.0) is added to the dried PNA oligomer-DSS, and this mixture is allowed to react overnight at room temperature. The resulting polyamine-PNA oligomer conjugate is characterized by reverse phase HPLC and a 20% denaturing gel. Solvent A is 50 mM TEAA, solvent B is CH_3CN. The HPLC gradient is from 0-10 mins, 95% solvent A, 5% solvent B; linear increase to 40% solvent B in the next 50 minutes using a Water's Delta-Pak C-18 reverse phase column.

HPLC analysis shows progressively faster migration times for the larger amines.

C. Preparation of Biotin Functionalized PNA Oligomer Polyamine Conjugate

       To further characterize the PNA-oligomer polyamine conjugate, biotin is attached to the free amines made available by the polyamines attached in the above Examples. About 10 O.D. units (A_{260}) of PNA oligomers SEQ ID NO:142 and SEQ ID NO:114, (approximately 58 nmoles) are dried in a microfuge tube.

5   The PNA oligomer polyamine conjugate is rehydrated in 400 ul of 0.2 M $NaHCO_3$ (pH 8.1) buffer and D-biotin-N-hydroxysuccinimide ester (approximately 5.0 mgs biotin for the 1,6 Diaminohexane conjugate, 8.0 mgs for the diethylenetriamine) (Sigma) is added followed by 200 ul of DMF. The solution is left to react overnight at room temperature. The solution is then passed over a NAP-25 column and analyzed by

10   reverse phase HPLC. Solvent A is 50 mM TEAA and solvent B is CH_3CN. The HPLC gradient wis 0-10 mins, 95% A, 5% B; linear increase to 40% B in the next 50 minutes using a Water's Delta-Pak C-18, reverse phase column.

EXAMPLE 96

15   **Internal Polyamine Labeled PNA Oligomer: linkage to formyl-functionalized monomer**

      1.   **N-[(N-Boc-1-formyl)-2-aminoethyl]-N-[(1-thyminyl)-acetyl]glycine ethyl ester (97A)**

       Compound **35** is converted to the ethyl ester by standard techniques. The

20   resulting compound is then treated according to the procedure of Example **37** to yield the title compound.

      2.   **N-[(N-Boc){1-(6-aminohexyl)aminomethyl}-2-aminoethyl]-N-[(1-thyminyl)-acetyl]glycine ethyl ester (97B)**

       Compound **97A** is dissolved in 100 microliters of water, and 100 ml of 1M

25   NaOAc buffer (pH 5.0) is added followed by 1,6 diaminohexane hydrochloride (24 μmols) and 50 μl of 60 mM $NaCNBH_3$ solution. The solution is vortexed, left overnight and purified in an analytical HPLC column. The product is then deprotected at the carboxyl group to yield the title compound (**97B**).

      3.   **Incorporation of N-[(N-Boc){1-(6-aminohexyl)aminomethyl}-2-**

30   **aminoethyl]-N-[(1-thyminyl)-acetyl]glycine ethyl ester (97B) into PNA oligomers.**

The free amino group of compound **97B** is Boc-protected using standard techniques. The protected monomer is then incorporated into PNA oligomers according to the procedure of Example **26**.

5   **EXAMPLE 97**

C-8 Purine and C-5 Pyrimidine conjugation

The C-8 position of purines and the C-5 position of pyrimidines are modified as per the procedures outlined in Ruth, J.L., Oligonucleotides with reporter groups attached to the base, *in Oligonucleotides and Analogues, A Practical Approach,* Eckstein, F., IRL

10   Press, New York.

A.   N-(N-Boc-2-aminoethyl)-N-[(N-6-Cbz)(8-[N-(6-trifluoroacetylaminohexyl)-3-(E)acrylamido]-9-adeninyl)acetyl]glycine (Compound 97A).

Compound **97A** is synthesized using the procedures of the above reference. A

15   purine substituted protected PNA monomer is used in place of a nucleoside. N-(N-Boc-2-aminoethyl)-N-[(6-Cbz-9-adeninyl)acetyl]glycine ethyl ester **(21)** is used as the starting material instead of adenosine. After the protected linking group is attached the resulting compound is treated via the procedures of Example 22 to give the title compound. The final compound having a protected linking moiety is further

20   incorporated into oligomers using standard methods. The protected linking moiety is deprotected and functionalized with a further functional group e.g. fluorescein or biotin.

B.   N-(N-Boc-2-aminoethyl)-N-[(N-4-Cbz)(5-[N-(6-trifluoroacetylaminohexyl)-3-(E)acrylamido]-1-cytosinyl)acetyl]glycine (Compound 97B).

25   Compound **97B** is synthesized using the procedures of the above reference except that Compound **16** is used as the starting material instead of cytidine. After the protected linking group is attached the resulting compound is treated via the procedures of Example **17**. The final compound having a protected linking moiety is further incorporated into oligomers using standard methods. The protected linking moiety is

30   deprotected and functionalized with a further functional group e.g. fluorescein or biotin.

EXAMPLE 98

5-Propynyl Pyrimidines (Cytosine and Uracil)

        A.   N-(N-Boc-2-aminoethyl)-N-[(N-4-Cbz)(5-propynyl)-1-cytosinyl)-

5   acetyl]glycine (Compound 98A)

        Compound **98A** is synthesized using the procedures of Wagner, W.W., *et.al.*,

Science, **1993**, *260*, 1510-1513, except that Compound **16** is used as the starting

material instead of cytidine. After the 5-propynyl group is attached to the protected N-1-

acetylcytosine the resulting compound is treated via the procedures of Example **17**. The

10   final compound having a 5-propynyl group is further incorporated into oligomers using

standard methods.

        B.   N-(N-Boc-2-aminoethyl)-N-[(5-propynyl)-1-urasinyl)acetyl]glycine

(Compound 98B)

        Compound **98B** is synthesized analogously to Compound **98A** except that the

15   protection step for the exocyclic amino group is omitted and uracil is substituted for

cytosine in the applicable examples e.g. Examples **13-17**. After the 5-propynyl group is

attached to N-1-acetyluracil the resulting compound is treated via the procedures of

Example **17**. The final compound having a 5-propynyl group is further incorporated into

oligomers using standard methods.

20

EXAMPLE 99

N-Fmoc-3-Aminopropionic Acid

        Sodium bicarbonate (2.52 g, 30 mmol) and 3-aminopropionic acid (1.00 g, 11.2

mmol) were dissolved in 50 ml water and 50 ml dioxane was added. A solution of

25   fluorenylmethyl chloroformate (3.10 g, 12.0 mmol) in 50 ml dioxane was added

dropwise with stirring. After 6 hours the solution was diluted with water (100 ml) and

saturated bicarbonate solution (50 ml), extracted once with diethyl ether, and the

aqueous layer acidified to pH 2 with concentrated HCl. The cloudy solution was

extracted with ethyl acetate (2 X 100 ml), washed with brine and dried with $MgSO_4$.

30   After evaporation a mixture of the title product and the peptide dimer was obtained. The

pure product was obtained by flash chromatography. ^{1}H NMR: (CDCl$_3$, 200 MHz) δ 7.95-7.26 (8H, m, ArH), 7.40-7.15 (3H, m, CHCH$_2$O),3.20 (2H, t, J=8 Hz, CH$_2$N), 2.40 (2H, t, J=8 Hz, HOOCCH$_2$).

5   **EXAMPLE 100**

N-(N-Boc-2-aminoethyl)-N-(N-Fmoc-3-aminopropionoyl)glycine (100)

This compound is prepared as per the procedures described in Examples **10** and **11** substituting N-Fmoc-3-aminopropionic Acid **(99)** in place of N-1-carboxymethyl thymine. The final compound is further incorporated into oligomers using standard

10   methods.

EXAMPLE 101

N-Imidazolyl-2-Acetic acid

Imidazole (3.7 g, 54 mmol) was added to a suspension of sodium hydride (2.6 g

15   of a 60% dispersion in oil, 60 mmol) in 50 ml dry THF. Bromoacetic acid (3.4 g, 24 mmol) was then added and the mixture stirred overnight. Water (1 ml) was then added and the solvent removed under reduced pressure. The residue was taken up in water (50 ml, pH >10), extracted with ether and the organic layer discarded. The aqueous layer was acidified to pH 1 with concentrated HCl and extracted again with ether. The

20   aqueous layer was evaporated to dryness. The oily residue was dissolved in absolute ethanol (EtOH) to precipitate NaCl, and recrystallized from acetone/methanol to give 1.22 g (7.5 mmol, 30%) pure product as the hydrochloride. ^{1}H NMR: (DMSO-d6, 200 MHz) δ 9.20 (s, H2), 7.76 (d, J = 1.5 Hz), 7.69 (d, J = 1.5 Hz), 5.20 (s, CH$_2$).0

25   **EXAMPLE 102**

N-(N-Boc-2-aminoethyl)-N-(N-Imidazolyl-2-Acetyl)glycine (102)

This compound is prepared as per the procedures described in Examples **10** and **11** substituting N-imidazolyl-2-acetic acid **(101)** in place of N-1-carboxymethyl thymine. The final compound is further incorporated into oligomers using standard

30   methods.

EXAMPLE 103

Benzyl 3,6,9,12-Tetraoxatridecanoate (103)

Triethyleneglycol monomethyl ether (10 mmol) and benzyl bromoacetate (11 mmol) are added to a suspension of anhydrous K_2CO_3 (15 mmol) in 50 ml anhydrous DMF. The suspension is stirred at room temperature overnight. Water is added and the emulsion is extracted with ethyl acetate (3 X 200 ml), washed with water, brine, and dried with $MgSO_4$. The solvent is evaporated and the residual oil purified by flash chromatography to give the title compound.

EXAMPLE 104

3,6,9,12-Tetraoxatridecanoic Acid (104)

Benzyl-3,6,9,12-Tetraoxatridecanoate (103, 5 mmol) is dissolved in methanol (50 ml) and 10% palladium on carbon is added (100 mg catalyst/mmol). The suspension is shaken under 30 psi H_2 until the starting material is consumed. The suspension is filtered through a short pad of Celite, washed thoroughly with methanol, and the solvent evaporated. The product is used directly without purification.

Example 105

N-(N-Boc-2-aminoethyl)-N-(3,6,9,12-Tetraoxatridecanoyl)glycine (105)

This compound is prepared as per the procedures described in Examples 10 and 11 substituting 3,6,9,12-tetraoxatridecanoic Acid (104) in place of N-1-carboxymethyl thymine. The final compound is further incorporated into oligomers using standard methods.

EXAMPLE 106

N-α-(FMOC)-glutamic acid γ-benzyl ester (106)

To a solution of γ-benzyl glutamate (10 mmol) in 50 ml dioxane and 50 ml water is added triethylamine (25 mmol), followed by a solution of fluorenylmethyl chloroformate (11 mmol) in 50 ml dioxane. The mixture is vigorously stirred until the starting material is consumed. The solution is acidified to pH 2 with concentrated HCl,

extracted with ethyl acetate (2 X 250 ml), washed with brine, dried with MgSO$_4$ and evaporated. The product is used without purification.

EXAMPLE 107

5   **N-α-(FMOC)-γ-benzyl-L-glutamic acid fluorenylmethyl ester (107)**

N-α-(FMOC)-glutamic acid γ-benzyl ester (**106**, 5 mmol), fluorenylmethanol (5.5 mmol) and dimethylaminopyridine (0.5 mmol) are dissolved in 50 ml CH$_2$Cl$_2$. Dimethylaminopropyl ethyl carbodiimide (EDC, 6.0 mmol) is added, and the solution stirred at room temperature. After complete consumption of the starting material the

10 solution is diluted with CH$_2$Cl$_2$, washed with 1% HCl, water and brine, dried with MgSO$_4$ and evaporated. The residue is purified by flash chromatography using ethyl acetate and hexane as eluant.

EXAMPLE 108

15 **N-α-(FMOC)-L-glutamic acid α-fluorenylmethyl ester (108)**

N-α-(FMOC)-γ-benzyl-L-glutamic acid fluorenylmethyl ester (**107**, 5 mmol) is dissolved in methanol (50 ml) and 10 % Palladium on carbon is added (100 mg catalyst/mmol). The suspension is shaken under 30 psi H$_2$ until the starting material is consumed. The suspension is filtered through a short pad of Celite, washed thoroughly

20 with methanol, and the solvent evaporated. The product is used directly without purification.

Example 109

N-(N-Boc-2-aminoethyl)-N-[N-α-(FMOC)-L-glutamoyl-α-fluorenylmethyl ester]-

25 **glycine (109)**

This compound is prepared as per the procedures described in Examples **10** and **11** substituting N-α-(FMOC)-L-glutamic acid α-fluorenylmethyl ester (**108**) in place of N-1-carboxymethyl thymine. The final compound is further incorporated into the N-terminus of PNA oligomers using standard methods.

30

EXAMPLE 110

N-(N-Boc-2-aminoethyl)-N-(1-methyl-2-pyrroleacetyl)glycine (110)

This compound is prepared as per the procedures described in Examples **10** and **11** substituting 1-methyl-2-pyrrolecarboxylic acid (Aldrich) in place of N-1-carboxy-methyl thymine. The final compound is further incorporated into oligomers using standard methods.

EXAMPLE 111

Cleavage of Plasmid-DNA by NTA-Lys$_2$-T$_2$CT$_2$CT$_4$-Lys$_2$-NH$_2$

A mixture of 2.0μL 5.9μM PNA NTA-Lys$_2$-T$_2$CT$_2$CT$_4$-Lys$_2$-NH$_2$ and 0.2μL 5.9μM Fe^{2+} was incubated for 10 minutes at room temperature. Buffer (100 mM PO$_4$, pH 6.5 or 100 mM PO$_4$, pH 6.5, 10 mM EDTA, 9.5 μL H$_2$O and 0.5 μL ^{32}P-labeled targeted DNA, pA8G2 was added and the incubation was continued for 1 hour at 37°C. 1μL of 40 mM DTT was added and the incubation continued for 1 hour at 37°C. Samples were analyzed on an 8% polyacrylamide sequencing gel according to the following scheme:

nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
PNA	+	+	+	+	+	+	+	+		+	+	+	+	+		
Fe^{2+}	+			+			+	+		+	+	+	+			
PNA/Fe^{2+}	10			10			10	10		0.5	1	4	10			
EDTA				+	+	+		+								
5'*	+	+	+	+	+	+	+	+	+							
3'*										+	+	+	+	+	+	+
DTT	+	+		+	+					+	+	+	+			
DMS							+	+	+							
$KMnO_4$														+	+	
seq.																+

5* denotes that the EcoR1/Pvu 11 fragment of pA8G2 was labeled at the 5'-end and 3* that this fragment was labeled at the 3'end. DMS denotes probing with dimethylsulphate and KMnO$_4$.

When the experimental results were analyzed by gel electrophoresis (see Figure

5   6) it was shown that PNA NTA Fe2$^+$ was an effective oligonucleotide cleavage agent. A comparison of lane 1 to lane 4 where EDTA was employed to competitively bind available Fe2$^+$ shows that the Fe2$^+$-NTA complex was necessary and effective at cleaving the DNA strand. A comparison of lane 13 to lane 14 shows that the cleaving pattern has been extended beyond the PNA binding site as mapped by KMnO4 cleavage

10   of the strand displaced by the PNA which is consistent with the Fe2$^+$-NTA complex being oriented at that end.

EXAMPLE 112

Binding of PNA conjugates to double stranded DNA targets

15   A mixture of 200 cps ^{32}P-labeled EcoRI-PvuII fragment (the large fragment labeled at the 3'-end of the EcoRI site) of the indicated plasmid, 0.5 µg carrier calf thymus DNA, and 300 ng PNA having an appropriate conjugate attached thereto in 100 µl buffer (10 mM Na-phosphate, 1mM EDTA pH 7) are incubated at 37°C for 60 min. 5 µl 20mM KMnO$_4$ is added and incubated at 20°C for 15 seconds. The reaction is

20   stopped by addition of 3 µl 0.5 M 2-mercaptoethanol, 1M NaOAc and the DNA is precipitated by addition of 250 µl 2% potassium acetate in ethanol. The DNA is treated with hot (50 °C) 1M piperidine and is analyzed by electrophoresis in 10% polyacrylamide sequencing gels and the radiolabeled DNA bands visualized by autoradiography.

25   The target plasmids are prepared by cloning of the appropriate oligonucleotides into pUC19. Target A10: oligonucleotides GATCCA10G & GATCCT10G cloned into the BamHI site (plasmid designated pT10). Target A5GA4: oligonucleotides TCGACT4CT5G & TCGACA5GA4G cloned into the SalI site (plasmid pT9C). Target A2GA2GA4: oligonucleotides GA2GA2GA4TGCA & GT4CT2CT2CTGCA into the

30   PstI site (plasmid pT8C2). The positions of the targets in the gel are indicated by bars to

the left. A/G is an A+G sequence ladder of target P10.

EXAMPLE 113

     A.   **Determination Of Cellular Uptake And Activity Of Thiol Linker**

Containing PNA Oligomers

     This is determined by the inhibition of ICAM-1 (Intra Cellular Adhesion

Molecule-1) utilizing the method of Chiang, *et al.*, *J. Biol. Chem.* **1991**, *266* 18162.

     ICAM-1 Assay: ICAM-1 expression on the cell surface is determined by ELISA

using cells grown to confluence in 96-well plates. Cells are washed three times with

Dulbecco's phosphate-buffered saline and fixed for 20 minutes in 2% formaldehyde

diluted in Dulbecco's PBS. The cells are washed three times with Dulbecco's PBS,

blocked for 1 hour at 37°C with 2% bovine serum albumin in Dulbecco's PBS, and

incubated with ICAM-1 monoclonal antibody 84H10 (0.5 µg/ml) for 1 hour at 37°C.

Antibody bound to the cells is determined by incubation with a 1:1000 dilution of

biotinylated goat anti-mouse IgG followed by incubation with a 1:1000 dilution of β-

galactosidase-conjugated streptavidin. The plates are developed with 100 µl of 3.3 mM

chlorophenolred-β-D-galactopyranoside in 50 mM sodium phosphate, 1.5 mM $MgCl_2$,

pH 7.0. Product formed is detected by absorbance at 575 nm. The data are expressed as

percent control activity, which is calculated as described by Chiang, *et al.*, in the above

reference.

     PNA Oligomer Treatment of Cells: Cells are washed three times with Opti-

MEM prewarmed to 37°C. Opti-MEM containing either 10 µg/ml DOTMA solution

(HUVEC) or 20 mg/ml DOTMA solution (A549 cells) is added to each well of the plate

(100 µl). PNA oligomers were sterilized by centrifugation through 0.2 µM Centrex

cellulose acetate filters. PNA oligomers are added as 20 X stock solution to the wells

and incubated for 4 hours at 37°C and then stimulated with the appropriate cytokine for

14-16 hours as indicated. ICAM-1 expression is determined as described above.

     The following PNA oligomers having aminoethylglycine backbones are

synthesized according to the procedures of the foregoing Examples: H_2N-TGG GA*G

CCA TAG CGA GCC-COOH (SEQ ID NO:147), ICAM PNA; and H_2N-TCT GAG

TAG CAG AGG AGC TA*A G (SEQ ID NO:148); a sequence in the 5'-cap region of

ICAM. The asterisk (*) denotes the incorporation of a monomeric unit containing a

protected thiol functionality according to Example **58**. PNA oligomer having SEQ ID

NO:117 serves to evaluate the tritylthioether group in uptake experiments and to

5   determine its ability to inhibit ICAM protein expression. PNA oligomer SEQ ID

NO:118 is conjugated to *O*-phenanthroline and targeted against the 5'-cap-messenger

RNA of the ICAM system to cleave the target RNA. The PNA oligomer is used in the

above assay to assess its effect on ICAM expression.

B.   RNA Cleavage Assay Using Oligonucleotide Containing Thiol Linker

10   PNA oligomer SEQ ID NO:118 is thiol deprotected and conjugated to *O*-

phenanthroline reagent. The conjugate is targeted against 5'-capped RNA of the ICAM

system. The hybrid is incubated at 37°C over a 48 hour period in the presence of excess

Cu(II) salt under buffered conditions. Analysis of the reaction by gel electrophoresis (as

described by Baker, *J. Am. Chem. Soc.* **1993**, *115*, 3378) demonstrates that the

15   oligonucleotide-*O*-phenanthroline-Cu complex cleaves the target RNA strand.

EXAMPLE 114

Inhibition of restriction enzyme cleavage by PNA-conjugate

A 2 μg portion of plasmid pT10 is mixed with the indicated amount of PNA

20   conjugate in 20 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) and incubated at

37°C for 120 min. 2 μl 10 × buffer (10 mM Tris-HCl, pH 7.5, 10 mM, MgCl$_2$, 50 mM

NaCl, 1 mM DTT). *Pvu*II (2 units) and *Bam*HI (2 units) are added and the incubation is

continued for 60 min. The DNA is analyzed by gel electrophoresis in 5%

polyacrylamide and the DNA is visualized by ethidium bromide staining.

25

EXAMPLE 115

Kinetics of PNA conjugate - dsDNA strand displacement complex formation

A mixture of 200 cps ^{32}P-labeled *Eco*RI-*Pvu*II fragment of pT10 (the large

fragment labeled at the 3'-end of the *Eco*RI site), 0.5 μg carrier calf thymus DNA, and

30   300 ng of PNA conjugate in 100 μl buffer (200 mM NaCl, 50 mM Na-acetate, pH 4.5, 1

mM $ZnSO_4$) are incubated at 37°C. Periodically, 50 U of S_1 nuclease is added to each

of 7 samples and incubation is continued for 5 min at 20°C. The DNA is then

precipitated by addition of 250 µl 2% K-acetate in ethanol and analyzed by

electrophoresis in a 10% polyacrylamide sequencing gel. The amount of strand

5   displacement complex is calculated from the intensity of the S_1-cleavage at the target

sequence, as measured by densitometric scanning of autoradiographs.

EXAMPLE 116

Stability of PNA-dsDNA complexes

10   A mixture of 200 cps ^{32}P-pT10 fragment, 0.5 µg calf thymus DNA and 300 ng of

the desired PNA conjugate is incubated in 100 µl 200 mM NaCl, 50 mM Na-acetate, pH

4.5, 1 mM $ZnSO_4$ for 60 min at 37°C. A 2 µg portion of oligonucleotide $GATCCA_{10}G$

is added and each sample is heated for 10 min at the temperature indicated, cooled in ice

for 10 min and warmed to 20°C. A 50 U portion of S_1 nuclease is added and the

15   samples treated and analyzed and the results quantified.

EXAMPLE 117

Inhibition of Transcription by PNA

A mixture of 100 ng plasmid DNA (cleaved with restriction enzyme *Pvu*II (see

20   below) and 100 ng of PNA conjugate in 15 µl 10 mM Tris-HCl, 1 mM EDTA, pH 7.4 is

incubated at 37°C for 60 min. Subsequently, 4 µl 5 × concentrated buffer (0.2 M Tris-

HCl (pH 8.0), 40 mM $MgCl_2$, 10 mM spermidine, 125 mM NaCl) are mixed with 1 µl

NTP-mix (10 mM ATP, 10 mM CTP, 10 mM GTP, 1 mM UTP, 0.1 µCi/µl ^{32}P-UTP, 5

mM DTT, 2 µg/ml tRNA, 1 µg/ml heparin) and 3 units RNA polymerase. Incubation is

25   continued for 10 min at 37°C. The RNA is then precipitated by addition of 60 µl 2%

potassium acetate in 96% ethanol at -20°C and analyzed by electrophoresis in 8%

polyacrylamide sequencing gels. RNA transcripts are visualized by autoradiography.

The following plasmids are used: pT8C2-KS/pA8G2-KS: oligonucleotides

$GA_2GA_2GA_4GTGAC$ & $GT_4CT_2CT_2CTGCA$ cloned into the *Pst*I site of pBluescript-

30   KS^+; pT10-KS/pA10-KS (both orientations of the insert were obtained). pT10UV5:

oligonucleotides GATCCA$_{10}$G & GATCCT$_{10}$G cloned into the *Bam*HI site of a pUC18 derivative in which the lac UV5 *E.coli* promoter had been cloned into the *Eco*RI site (Jeppesen, *et al.*, *Nucleic Acids Res.*, **1988**, *16*, 9545).

5   **EXAMPLE 118**

Biological stability of PNA

A mixture of PNA conjugate (10 µg) and a control, "normal" peptide (10 µg) in 40 µl 50 mM Tris-HCl, pH 7.4 is treated with varying amounts of peptidase from porcine intestinal mucosa or protease from Streptomyces caespitosus for 10 min at

10   37°C. The amount of PNA and peptide is determined by HPLC analysis (reversed phase C-18 column: 0-60% acetonitrile, 0.1% trifluoroacetic acid or other appropriate solvent system).

EXAMPLE 119

15   **Inhibition of Gene Expression**

A preferred assay to test the ability of peptide nucleic acids to inhibit expression of the E2 mRNA of papillomavirus is based on the well-documented transactivation properties of E2. Spalholtz, *et al.*, *J. Virol.*, **1987**, *61*, 2128-2137. A reporter plasmid (E2RECAT) is constructed to contain the E2 responsive element, which functions as an

20   E2 dependent enhancer. E2RECAT also contains the SV40 early promoter, an early polyadenylation signal, and the chloramphenicol acetyl transferase gene (CAT). Within the context of this plasmid, CAT expression is dependent upon expression of E2. The dependence of CAT expression on the presence of E2 is tested by transfection of this plasmid into C127 cells transformed by BPV-1, uninfected C127 cells and C127 cells

25   cotransfected with E2RECAT and an E2 expression vector.

A.   Inhibition of BPV-1 E2 Expression

BPV-1 transformed C127 cells are plated in 12 well plates. Twenty four hours prior to transfection with E2RE1, cells are pretreated by addition of complementary PNA conjugates to the growth medium at final concentrations of 5, 15 and 30 mM. The

30   next day cells are transfected with 10 µg of E2RE1CAT by calcium phosphate

precipitation. Ten micrograms of E2RE1CAT and 10 μg of carrier DNA (PUC 19) are mixed with 62 μl of 2 M CaCl$_2$ in a final volume of 250 μl of H$_2$0, followed by addition of 250 μl of 2X HBSP (1.5 mM Na$_2$PO$_2$. 10 mM KCl, 280 mM NaCl, 12 mM glucose and 50 mM HEPES, pH 7.0) and incubated at room temperature for 30 minutes. One

5   hundred microliters of this solution is added to each test well and allowed to incubate for 4 hours at 37°C. After incubation, cells are glycerol shocked for 1 minute at room temperature with 15% glycerol in 0.75 mM Na$_2$PO$_2$, 5 mM KCl, 140 mM NaCl, 6 mM glucose and 25 mM HEPES, pH 7.0. After shocking, cells are washed 2 times with serum free DMEM and refed with DMEM containing 10% fetal bovine serum and

10   oligonucleotide at the original concentration. Forty eight hours after transfection cells are harvested and assayed for CAT activity.

For determination of CAT activity, cells are washed 2 times with phosphate buffered saline and collected by scraping. Cells are resuspended in 100 μl of 250 mM Tris-HCl, pH 8.0 and disrupted by freeze-thawing 3 times. Twenty four microliters of

15   cell extract is used for each assay. For each assay the following are mixed together in an 1.5 ml Eppendorf tube and incubated at 37°C for one hour: 25 μl of cell extract, 5 μl of 4 mM acetyl coenzyme A, 18 μl H$_2$O and 1 μl ^{14}C-chloramphenicol, 40-60 mCi/mM. After incubation, chloramphenicol (acetylated and nonacetylated forms) is extracted with ethyl acetate and evaporated to dryness. Samples are resuspended in 25 μl of ethyl

20   acetate, spotted onto a TLC plate and chromatographed in chloroform:methanol (19:1). Chromatographs are analyzed by autoradiography. Spots corresponding to acetylated and nonacetylated ^{14}C-chloramphenicol are excised from the TLC plate and counted by liquid scintillation for quantitation of CAT activity. Peptide nucleic acids that depress CAT activity in a dose dependent fashion are considered positives.

25   **B.   Inhibition of HPV E2 Expression**

The assay for inhibition of human papillomavirus (HPV) E2 by peptide nucleic acids is essentially the same as that for BPV-1 E2. For HPV assays appropriate HPVs are co-transfected into either CV-1 or A431 cells with PSV2NEO using the calcium phosphate method described above. Cells which take up DNA are selected for by

30   culturing in media containing the antibiotic G418. G418-resistant cells are then

analyzed for HPV DNA and RNA. Cells expressing E2 are used as target cells for complementary studies. For each PNA, cells are pretreated as above, transfected with E2RE1CAT, and analyzed for CAT activity as above. Peptide nucleic acid conjugates are considered to have a positive effect if they can depress CAT activity in a dose

5 dependent fashion.

Example 120

Iodination Procedure

A 5 µg portion of a PNA having a conjugate attached thereto is dissolved in 40

10 µl 100 mM Na-phosphate, pH 7.0, and 1 mCi Na^{125}I and 2 µl chloramine-T (50 mM in CH$_3$CN) are added. The solution is left at 20°C for 10 min and then passed through a 0.5 + 5 cm Sephadex G10 column. The first 2 fractions (100 µl each) containing radioactivity are collected and purified by HPLC: reversed phase C-18 using a 0-60% CH$_3$CN gradient in 0.1% CF$_3$COOH in H$_2$O, or other suitable solvent system. The ^{125}I-

15 PNA elutes right after the PNA peak. The solvent is removed under reduced pressure.

EXAMPLE 121

Detection of mutant ß-amyloid precursor protein gene expression (ßAPP)

Point mutations in the gene encoding ß-amyloid have been implicated in familial

20 Alzheimer's disease (FAD). PNA oligomers are labeled after synthesis with fluorescein or other fluorescent tag as illustrated above. Alternatively,oligomers can be labeled with other reporter molecules before or after oligomer synthesis. Labeled PNA oligomers are contacted with tissue or cell samples suspected of abnormal ßAPP expression under conditions in which specific hybridization can occur, and the sample is washed to

25 remove unbound PNA oligomers. Label remaining in the sample indicates bound oligonucleotide and is quantitated using a fluorimeter, fluorescence microscope or other routine means.

Tissue or cell samples suspected of expressing a point mutation in the ßAPP gene are incubated with a fluorescein-labeled PNA oligomer which is targeted to the

30 mutant codon 717, codon 670 or codon 671 of ßAPP mRNA. An identical sample of

cells or tissues is incubated with a second labeled PNA oligomer which is targeted to the same region of normal ßAPP mRNA, under conditions in which specific hybridization can occur, and the sample is washed to remove unbound PNA oligomer. Label remaining in the sample indicates bound PNA oligomer and can be quantitated using a

5   fluorimeter or other routine means. The presence of mutant ßAPP is indicated if the first sample binds labeled PNA oligomer and the second sample does not bind fluorescent label.

Double labeling can also be used with PNA oligomers and methods of the invention to specifically detect expression of mutant ßAPP. A single tissue sample is

10   incubated with a rhodamine-labeled PNA oligomer which is targeted to codon 717, codon 670 or codon 671 of mutant ßAPP mRNA and a fluorescein-labeled PNA oligomer which is targeted to the translation initiation site of mutant ßAPP mRNA, under conditions in which specific hybridization can occur. The sample is washed to remove unbound PNA oligomer and labels are detected by and fluorimetry with

15   appropriate filters. The presence of mutant ßAPP is indicated if the sample does not bind rhodamine-labeled PNA oligomer but does retain the fluorescein label.

EXAMPLE 122

Detection of mutant H-ras gene expression

20   Point mutations in the H-ras gene have been implicated in numerous aberrations of the Ras pathway. PNA oligomers are labeled after synthesis with fluorescein or other fluorescent tag as illustrated above. Alternatively, oligomers can be labeled with other reporter molecules before or after oligomer synthesis. Labeled PNA oligomers are contacted with tissue or cell samples suspected of abnormal ras expression under

25   conditions in which specific hybridization can occur, and the sample is washed to remove unbound PNA oligomer. Label remaining in the sample indicates bound PNA oligomer and is quantitated using a fluorimeter, fluorescence microscope or other routine means.

Tissue or cell samples suspected of expressing a point mutation in the H-ras gene

30   are incubated with a fluorescein-labeled PNA oligomer which is targeted to the mutant

codon 12, codon 13 or codon 61 of H-ras mRNA. An identical sample of cells or tissues is incubated with a second labeled PNA oligomer which is targeted to the same region of normal H-ras mRNA, under conditions in which specific hybridization can occur, and the sample is washed to remove unbound PNA oligomer. Label remaining in the sample

5   indicates bound PNA oligomer and can be quantitated using a fluorimeter or other routine means. The presence of mutant H-ras is indicated if the first sample binds labeled PNA oligomer and the second sample does not bind fluorescent label.

Double labeling can also be used with PNA oligomers and methods of the invention to specifically detect expression of mutant ras. A single tissue sample is

10   incubated with a rhodamine-labeled PNA oligomer which is targeted to codon 12, codon 13 or codon 61 of mutant H-ras mRNA and a fluorescein-labeled PNA oligomer which is targeted to the translation initiation site of ras mRNA, under conditions in which specific hybridization can occur. The sample is washed to remove unbound PNA oligomer and labels are detected by and fluorimetry with appropriate filters. The

15   presence of mutant ras is indicated if the sample does not bind rhodamine-labeled PNA oligomer but does retain the fluorescein label.